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β-Cryptoxanthin Stimulates Apoptotic Cell Death and Suppresses Cell Function in Osteoclastic Cells: Change in Their Related Gene Expression

Satoshi Uchiyama and Masayoshi Yamaguchi*

Laboratory of Endocrinology and Molecular Metabolism, Graduate School of Nutritional Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan

Abstract The effect of β-cryptoxanthin, a kind of carotenoid, on osteoclastic cells in mouse marrow culture system in vitro was investigated. 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 NF-κB ligand (RANKL; 25 ng/ml) for 4 days. The osteoclastic cells formed were further cultured in medium containing either vehicle or β-cryptoxanthin (10^{-8}–10^{-6} M) with or without M-CSF (10 ng/ml) and RANKL (50 ng/ml) for 24–72 h. Osteoclastic cells were significantly decreased with culture of β-cryptoxanthin (10^{-7} or 10^{-6} M) with or without M-CSF and RANKL for 24, 48, or 72 h. β-Cryptoxanthin (10^{-8} M)-induced decrease in osteoclastic cells were significantly inhibited in the presence of caspase-3 inhibitor (10^{-8} or 10^{-7} M). Agarose gel electrophoresis showed the presence of low-molecular-weight deoxyribonucleic acid (DNA) fragments of adherent cells cultured with β-cryptoxanthin (10^{-7} or 10^{-6} M) for 24 or 48 h, indicating that the carotenoid induces apoptotic cell death. Apoptosis-related gene expression was determined using reverse transcription-polymerase chain reaction (RT-PCR). Culture with β-cryptoxanthin (10^{-7} or 10^{-6} M) for 24 or 48 h caused a significant increase in caspase-3 mRNA expression in the presence or absence of M-CSF and RANKL, while Bcl-2 and Apaf-2 mRNA expressions were significantly increased with culture of β-cryptoxanthin (10^{-7} or 10^{-6} M) without M-CSF and RANKL for 24 or 48 h. Akt-1 mRNA expression was not significantly changed with culture of the carotenoid (10^{-7} or 10^{-6} M) for 24 or 48 h. Moreover, tartrate-resistant acid phosphatase (TRACP) activity, or TRACP and cathepsin K mRNA expressions were significantly decreased with culture of β-cryptoxanthin (10^{-6} M) in the presence or absence of M-CSF and RANKL for 48 h. This study demonstrates that β-cryptoxanthin has stimulatory effects on apoptotic cell death and suppressive effects on osteoclastic cell function. J. Cell. Biochem. 98: 1185–1195, 2006.

Key words: β-cryptoxanthin; osteoclast; apoptosis; caspase-3; Bcl-2; TRACP; cathepsin K

Osteoporosis and 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. Pharmacologic and nutritional factors may prevent bone loss with increasing age [Bonjour et al., 1996]. The chemical compounds in food that act on bone metabolism, however, are poorly understood.

Retinol (vitamin A) is known to have a detrimental effect on bone at high doses. In laboratory animals, high levels of vitamin A lead to accelerated bone resorption, bone fractures, and osteoporotic bone lesions. [Forsyth et al., 1989; Rohde et al., 1999; Promislow et al., 2002]. The effects of carotenoids on bone metabolism, however, have not been fully clarified. Carotenoids are present in fruit and vegetables. Recent studies have shown that β-cryptoxanthin, a kind of carotenoid, has unique anabolic effects on bone calcification in vitro [Yamaguchi and Uchiyama, 2003]. Lutein, lycopene, and β-carotene, which are carotenoids, do not have an effect on bone calcification in femoral tissue culture in vitro [Yamaguchi and Uchiyama, 2003]. β-Cryptoxanthin has direct stimulatory effects on bone formation and inhibitory effects on bone resorption in cultured bone tissues in vitro [Yamaguchi and Uchiyama, 2004].

*Correspondence to: Masayoshi Yamaguchi, PhD, Laboratory of Endocrinology and Molecular Metabolism, Graduate School of Nutritional Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan.
E-mail: yamaguch@u-shizuoka-ken.ac.jp

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The oral administration of β-cryptoxanthin has been shown to induce anabolic effects on bone components in the femoral-diaphyseal (cortical bone) and -metaphyseal (trabecular bone) tissues in young and aged rats in vivo [Uchiyama et al., 2004a,b]. Streptozotocin-induced bone loss in rats with diabetic state is prevented by the oral administration of β-cryptoxanthin in vivo [Uchiyama and Yamaguchi, 2005a]. The oral administration of β-cryptoxanthin prevents bone loss in ovariectomized rats [Uchiyama and Yamaguchi, 2006]. Moreover, the prolonged intake of dietary β-cryptoxanthin has been shown to have stimulatory effects on bone formation and inhibitory effects on bone resorption by estimating serum biochemical markers of bone metabolism in healthy individuals [Yamaguchi et al., 2004]. β-Cryptoxanthin may have preventive effects on osteoporosis with increasing age.

β-Cryptoxanthin has been shown to have stimulatory effects on proliferation, differentiation, and mineralization by enhancing their gene expression of proteins [including Runx2 type 1 and type 2, alkaline phosphatase, and α 1(I) collagen], which are involved in bone formation in osteoblastic MC3T3-E1 cells [Uchiyama and Yamaguchi, 2005b,c]. Moreover, β-cryptoxanthin has been demonstrated to have potent inhibitory effects on osteoclast-like cell formation in mouse marrow culture in vitro [Uchiyama and Yamaguchi, 2004]. Thus the cellular mechanism by which β-cryptoxanthin has stimulatory effects on osteoclastic bone formation and inhibitory effects on osteoclastic bone resorption has been shown. This, however, remains to be elucidated.

The present study was undertaken to determine the effect of β-cryptoxanthin on apoptotic cell death and related gene expression in osteoclast-like cells formed in mouse marrow culture system in vitro. We found that culture with β-cryptoxanthin induces apoptotic cell death, which is related gene expression in osteoclastic cells.

MATERIALS AND METHODS

Chemicals

α-Minimal essential medium (α-MEM) and penicillin–streptomycin (5,000 U/ml penicillin; 5,000 μg/ml streptomycin) were obtained from Gibco Laboratories (Grand Island, NY). Fetal bovine serum (FBS), macrophage colony-stimulating factor (M-CSF, mouse), and receptor activator of NF-κB ligand (RANKL, mouse) were obtained from Sigma (St. Louise, MO). β-Cryptoxanthin was obtained from Extrasynthese (Lyon-Nord, France). Caspase-3/CPP 32 inhibitor W-1 (caspase-3 inhibitor) and other chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan). All water used were glass distilled.

Animals

Male mice (ddY strain; 6 weeks old) were obtained from Japan SCL (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 killed by exsanguinations.

Marrow Culture and Osteoclast Differentiation

Bone marrow cells were isolated from mice for studies on primary osteoclast precursor, as reported elsewhere [Ogasawara et al., 2004]. Briefly, 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 atomosphere containing 5% CO_2 and 95% air at 37°C. After 2 days, adherent cells were used as the M-CSF-dependent bone marrow macrophage (m-BMMø) after washing out the nonadherent 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.

To determine the effect of β-cryptoxanthin on osteoclastogenesis, the M-BMMø were further cultured with medium containing either vehicle (1% ethanol) or β-cryptoxsanthin (10^{-8}–10^{-6} M) in the presence of M-CSF (10 ng/ml) and RANKL (50 ng/ml) for 2 or 4 days.

To determine the effect of β-cryptoxanthin on the cell death and apoptosis in mature osteoclasts were incubated for 24, 48, or 72 h in medium containing either vehicle (1% ethanol) or β-cryptoxanthin (10^{-8}–10^{-6} M) in the presence or absence of M-CSF (10 ng/ml) and RANKL (25 ng/ml).

Enzyme Histochemistry

After culture, cells adherent to 24-well plates were stained for tartrate-resistant acid phosphatase (TRACP), a marker enzyme of osteoclasts [Burstone, 1958; Minkin, 1982]. Briefly, 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 [1958]. 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 48 h in medium containing either vehicle or β-cryptoxanthin (10^{-8}–10^{-6} M) with or without M-CSF (10 ng/ml) and RANKL (50 ng/ml). The culture supernatant was removed, and adherent cells were than lysed in 10 mM Tris-Hcl, pH 2.4, 10 mM EDTA (neutralized), and 0.5% Trion X-100. Low-molecular-weight DNA fragments were separated by electrophoresis in 1.5% agarose gel [Preaux et al., 2002]. Gels were visualized by ethidium bromide staining with an UV transilluminator (Funakoshi Co. Ltd., Tokyo, Japan). DNA content in the cell lysate was determined by the method of Ceriotti [1995].

Determination of Specific mRNA by RT-PCR

Total RNAs were prepared as described previously [Chomczyshi and Sacchi, 1987]. After the M-BMMø 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 48 h in medium containing either vehicle or β-cryptoxanthin (10^{-7} or 10^{-6} 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*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 diethylpyrocarbonate-treated water.

Reverse transcription-polymerase chain reaction (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 Apaf-1 cDNA were; 5′-AAGGTGGAGTACCCCAGAGGCCG-3′ (sense strand, positions 899-921 of cDNA sequence and 5′-GTCCAAGCGCATGCACAGATTCTG-3′ (antisense strand, positions 1,150–1,173) [Cecconi et al., 1998]. The pair of oligonuleotide primers was designed to amplify a 275-bp sequence from the mRNA of mouse Apaf-1. Primers for amplification of mouse caspase-3 cDNA were: 5′-GCTCTGGTACGGATGTGGACGCA-3′ (sense strand, positions 254–276 of cDNA sequence) and 5′-CTCAATGCCACAGTCCAGCTCCG-3′ (antisense strand, positions 560–582) [Juan et al., 1996]. The pair of oligonucleotide primers was designed to amplify a 329-bp sequence from the mRNA of mouse caspase-3. Primers for amplication of mouse Bcl-2 cDNA were; 5′-GATGACTTCTCTCGTCGCTACCGTCG-3′ (sense strand, positions 1,492–1,517 of cDNA sequence) and 5′-AGCAGGGTCTTCAGAGACACAGCCAGG-3′ (antisense strand, positions 1,826–1,850) [Negrini et al., 1987]. The pair of oligonucleotide primers was designed to amplity a 359-bp sequence from the mRNA of mouse Bcl-2. Primers for amplication of mouse Akt-1 cDNA were; 5′-GGAGGGCTGGCTGCACAAACG-3′ (sense strand, positions 307–327) and 5′-TCGTTCATGGTCACACGGTGCTTG-3′ (antisense strand, positions 706–729) [Bellacosa et al., 1993]. The pair of oligonucleotide primers was designed to amplify a 423-bp sequence from the mRNA of mouse Akt-1. Primers for amplication of mouse TRACP cDNA were; 5′-CAACGGCTACTTGCGGTTTC-3′ (sense strand, positions 963–982) and 5′-TGTGGGATCAGTTGGTGTGG-3′ (antisense strand, positions 1,281–1,300) [Cassady et al., 1993]. The pair of oligonucleotide primers was designed to amplify a 338-bp sequence from the mRNA of mouse TRACP. Primers for amplication of mouse cathepsin K cDNA were; 5′-CAGCAGGATGTGGGTGTTCA-3′ (sense strand, positions 47–66) and 5′-ACACTGGCCCTGGTTCTTGA-3′ (antisense strand, positions 442–461)

[Rantakokko et al., 1996]. The pair of oligonucleotide primers was designed to amplify a 415-bp sequence from the mRNA of mouse cathepsin K. For semiquantitative PCR, glyceroaldehyde-3-phosphate dehydrogenase (G3PDH) was used as an internal control to evaluate total RNA input. Primers for amplication 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 reaction mixture (20 μl) containing 4 μg of total RNAs, supplied RT-TCR buffer, Titam™ enzyme mix (AMV and Expand™ High Fidelity), 0.2 mM dNTP, 5 mM dithiothreitol, 5 U RNase inhibitor, and 0.3 μM primers. Samples were incubated at 50°C for 30 min, and then amplified for 35 cycles under the following conditions: denaturation for 30 s at 94°C, annealing for 30 s at 60°C, and extension for 60 s 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 with a Fluoro Imager SI (Amersham Pharmacia Biotech).

TRACP Activity Assay

The cultures with osteoclastic cells were washed with phosphate-buffered saline. Cell extracts were collected and disrupted with an ultrasonic device. Protein concentration and TRACP activity were assayed in the extracts. Protein concentration was assayed using the method of Lowry et al. [1951]. TRACP enzyme activity in cell extracts was assayed using *p*-nitrophenylphosphate (pNPP) as sustrate in an incubation medium (1.2 ml) containing the following: 10 mM pNPP, 0.1 M sodium acetate (pH 5.8), 0.15 MKCl, 10 mM sodium tartrate, 1 mM ascorbic acid, and 0.1 mM $FeCl_3$ [Minkin, 1982; Perez-Amodio et al., 2005]. The enzyme reaction mixture was incubated for 60 min at 37°C. After incubation, the reaction was stopped by the addition of 0.1 N NaOH (4.0 ml). The absorbance of *p*-nitrophenol liberated was immediately measured at 405 nm. TRACP activity was expressed as nmoles of *p*-nitrophenol liberated per minute per milligram of protein.

Statistical Analysis

Data are expressed as the mean ± SEM. Statistical differences were analyzed using Student's *t*-test. *P*-value less than 0.05 was considered to indicate a statistically significant difference. Also, we used an ANOVA multiple comparison test to compare the treatment groups.

RESULTS

Effect of β-Cryptoxanthin on Osteoclastic Cell Death

The effect of β-cryptoxanthin on osteoclast-like MNC formation induced in the presence of M-CSF and RANKL in the mouse marrow culture was examined. Osteoclast-like cells were markedly increased with culture in the presence of M-CSF (10 ng/ml) and RANKL (50 ng/ml) for 48 or 96 h (Fig. 1). This increase was significantly inhibited in the presence of β-cryptoxanthin (10^{-7} or 10^{-6} M), indicating that the carotenoid has a suppressive effect on osteoclastogenesis in the mouse marrow culture.

After the M-CSF-dependent bone marrow culture, macrophages were cultured in the presence of M-CSF (10 ng/ml) and RANKL (50 ng/ml) for 4 days, the adherent cells were further cultured in medium containing either vehicle (1% ethanol) or β-cryptoxanthin (10^{-8}–10^{-6} M) in the presence (Fig. 2) or absence

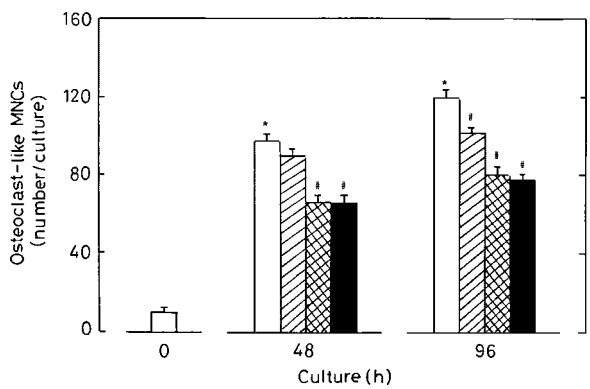

Fig. 1. Inhibitory Effect of β-cryptoxanthin on osteoclast-like cell formation in mouse marrow culture in the presence of M-CSF and RANKL. Mouse marrow cells were cultured as described in the Materials and Methods. The adherent cells were cultured for 48 or 96 h in medium containing either vehicle (1% ethanol) or β-cryptoxanthin (10^{-8}–10^{-6} M) in the presence of 0.5% FBS, M-CSF (10 ng/ml), and RANKL (50 ng/ml). Cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. Each value is the mean ± SEM of six cultures. $^{*}P < 0.01$ compared with the control (none) value at zero time. $^{\#}P < 0.01$ compared with the control (none) value at 48 or 96 h. White bars, control (none); hatched bars, β-cryptoxanthin (10^{-8} M); double hatched bars, β-cryptoxanthin (10^{-7} M); black bars, β-cryptoxanthin (10^{-6} M).

(Fig. 3) of M-CSF (10 ng/ml) and RANKL (50 ng/ml) for 24, 48, or 72 h. The formed osteoclastic cells were significantly decreased with culture for 48 or 72 h in the presence of M-CSF and RANKL as compared with the value obtained at the zero time (Fig. 2). This decrease was further enhanced in the absence of M-CSF and RANKL (Fig. 3). Cell death and apoptosis of osteoclastic cells were induced with longer periods of culture. The presence of β-cryptoxanthin (10^{-7} or 10^{-6} M) caused a significant decrease in osteoclastic cells in the presence (Fig. 2) or absence (Fig. 3) of M-CSF and RANKL as compared with the control value obtained at 24, 48, or 72 h after culture without the carotenoid.

The effect of caspase inhibitor on β-cryptoxanthin-induced cell death was examined (Fig. 4). Osteoclastic cells were cultured for 24 h in medium containing either vehicle (1% ethanol) or β-cryptoxanthin (10^{-7} or 10^{-6} M) with or without caspase inhibitor (10^{-8} or 10^{-7} M) in the absence of M-CSF and RANKL. The β-cryptoxanthin (10^{-7} M)-induced decrease in osteoclastic cells was significantly inhibited with culture in the presence of caspase inhibitor (10^{-8} or 10^{-7} M).

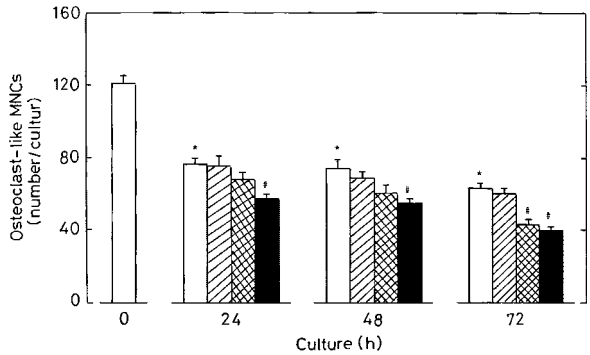

Fig. 3. Effect of β-cryptoxanthin on osteoclastic cell death in the absence of M-CSF and RANKL in mouse marrow culture. Mouse marrow cells were cultured as described in the Materials and Methods. The adherent cells were cultured for 96 h in medium containing 0.5% FBS, M-CSF (10 ng/ml), and RANKL (25 ng/ml). After medium change, the TRACP-positive MNCs formed were cultured for 24, 48, or 72 h in medium containing either vehicle (1% ethanol) or β-cryptoxanthin (10^{-8}–10^{-6} M) in the absence of M-CSF (10 ng/ml) plus RANKL (50 ng/ml). Cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. Each value is the mean ± SEM of six cultures. $^{*}P < 0.01$ compared with the control (none) value at zero time. $^{\#}P < 0.01$ compared with the control (none) value at 24, 48, or 72 h. White bars, control (none); hatched bars, β-cryptoxanthin (10^{-8} M); double hatched bars, β-cryptoxanthin (10^{-7} M); black bars, β-cryptoxanthin (10^{-6} M).

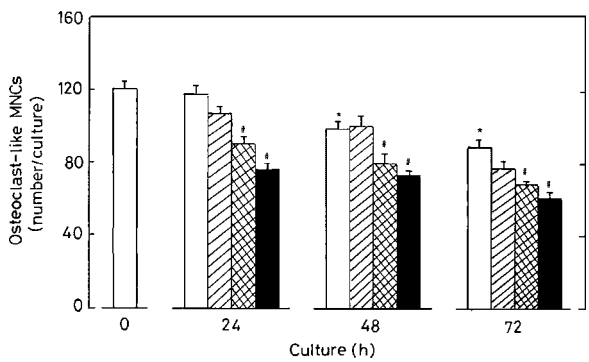

Fig. 2. Effect of β-cryptoxanthin on osteoclastic cell death in the presence of M-CSF and RANKL in mouse marrow culture. Mouse marrow cells were cultured as described in the Materials and Methods. The adherent cells were cultured for 96 h in medium containing 0.5% FBS, M-CSF (10 ng/ml), and RANKL (50 ng/ml). After medium change, the TRACP-positive MNCs formed were cultured for 24, 48, or 72 h in medium containing either vehicle (1% ethanol) or β-cryptoxanthin (10^{-8}–10^{-6} M) in the presence of M-CSF (10 ng/ml) plus RANKL (50 ng/ml). Cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. Each value is the mean ± SEM of six cultures. $^{*}P < 0.01$ compared with the control (none) value at zero time. $^{\#}P < 0.01$ compared with the control (none) value at 24, 48, or 72 h. White bars, control (none); hatched bars, β-cryptoxanthin (10^{-8} M); double hatched bars, β-cryptoxanthin (10^{-7} M); black bars, β-cryptoxanthin (10^{-6} M).

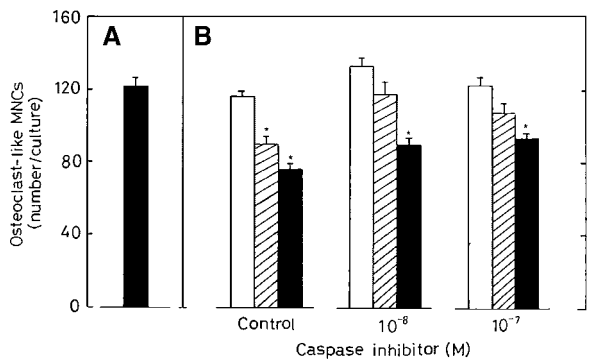

Fig. 4. Effect of β-cryptoxanthin in the presence of caspase inhibitor on osteoclastic cells formed in mouse marrow culture. Mouse marrow cells were cultured as described in the Materials and Methods. The adherent cells were cultured for 96 h in medium containing 0.5% FBS, M-CSF (10 ng/ml), and RANKL (50 ng/ml) (**A**). After medium change, the TRACP-positive MNCs formed were cultured for 24 h in medium containing either vehicle (1% ethanol) or β-cryptoxanthin (10^{-7} or 10^{-6} M) with or without caspase inhibitor (10^{-8} or 10^{-7} M) in the absence of M-CSF (10 ng/ml) plus RANKL (50 ng/ml) (**B**). Cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. Each value is the mean ± SEM of six cultures. $^{*}P < 0.01$ compared with the control (none) value with or without caspase inhibitor. White bars, without β-cryptoxanthin; hatched bars, with β-cryptoxanthin (10^{-7} M); black bars, with β-cryptoxanthin (10^{-6} M).

The effect of β-cryptoxanthin on DNA fragmentation in osteoclastic cells formed with culture in the presence of M-CSF and RANKL is shown in Figure 5. Osteoclastic cells were cultured for 24 or 48 h in medium containing either vehicle (1% ethanol) or β-cryptoxanthin (10^{-8}–10^{-6} 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. The presence of β-cryptoxanthin (10^{-7} or 10^{-6} M) caused DNA fragmentation in the presence or absence of M-CSF and RANKL.

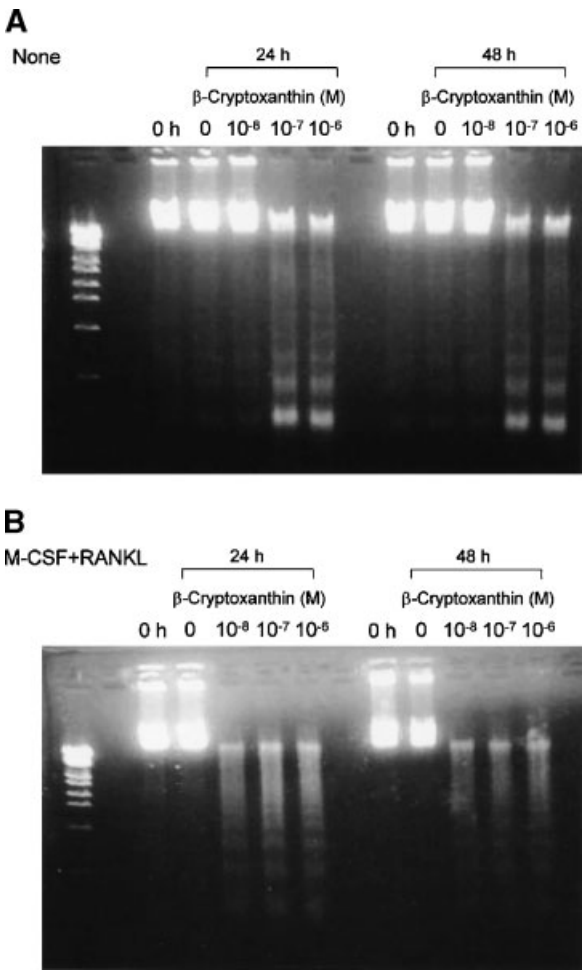

Fig. 5. Effect of β-cryptoxanthin on deoxyribonucleic acid (DNA) fragmentation in osteoclastic cell formed in mouse marrow culture. Mouse marrow cells were cultured as described in the Materials and Methods. The adherent cells were cultured for 96 h in medium containing 0.5% FBS, M-CSF (10 ng/ml), and RANKL (50 ng/ml). After medium change, the osteoclast-like cells formed were cultured for 0, 24, or 48 h in medium containing either vehicle (1% ethanol) or β-cryptoxanthin (10^{-8}–10^{-6} M) in the absence or presence of M-CSF (10 ng/ml) plus RANKL (50 ng/ml), and the lysate (containing DNA 2.0 μg) of adherent cells was applied to agarose gel. The figure shows one of five experiments with separate samples.

Effect of β-Cryptoxanthin on Apoptosis-Related Gene Expression in Osteoclastic Cells

The change in apoptosis-related gene expression in osteoclastic cells was investigated to determine possible mechanism by which β-cryptoxanthin suppresses cell death. The M-CSF-dependent bone marrow macrophages were cultured in the presence of M-CSF (10 ng/ml) and RANKL (50 ng/ml) for 4 days, and the adherent cells were further cultured in medium containing either vehicle (1% ethanol) or β-cryptoxanthin (10^{-7} or 10^{-6} M) in the presence or absence of M-CSF (10 ng/ml) and RANKL (50 ng/ml) for 24 or 48 h. The alteration in Apaf-2, caspase-3, Bcl-2, and Akt-1 mRNA expressions in osteoclast-like cells was examined. These proteins are related to apoptosis. Culture with β-cryptoxanthin (10^{-7} or 10^{-6} M) in the absence of M-SCF and RANKL for 24 or 48 h caused a significant increase in the expression of Apaf-2 and caspase-3 mRNAs (Fig. 6A,B). Caspase-3 mRNA expression was significantly increased with culture of β-cryptoxanthin (10^{-6} M) for 24 or 48 h in the presence of M-CSF and RANKL (Fig. 6B). The carotenoid did not have an effect on Apaf-2 mRNA expression (Fig. 6A). The expression of G3PDH mRNA was not significantly changed with culture of β-cryptoxanthin (10^{-7} or 10^{-6} M) in the presence or absence of M-CSF and RANKL (Fig. 6C). The expression of Bcl-2 mRNA was significantly decreased with culture of β-cryptoxanthin (10^{-6} M) in the presence or absence of M-CSF and RANKL, while Akt-1 mRNA expression was not significantly changed with the carotenoid (10^{-7} or 10^{-6} M) (Fig. 7).

Effect of β-Cryptoxanthin on TRACP Activity and Related Gene Expression in Osteoclastic Cells

The effect of β-cryptoxanthin on TRACP activity and its related gene expression was examined to determine whether the carotenoid has suppressive effects on osteoclastic cell function. The effect of β-cryptoxanthin on TRACP activity in osteoclastic cells is shown in Figure 8. The M-CSF-dependent bone marrow macrophages were cultured in the presence of M-CSF (10 ng/ml) and RANKL (50 ng/ml) for 4 days, and the adherent cells were further cultured in medium containing either vehicle or β-cryptoxanthin (10^{-7} or 10^{-6} M) in the pre-

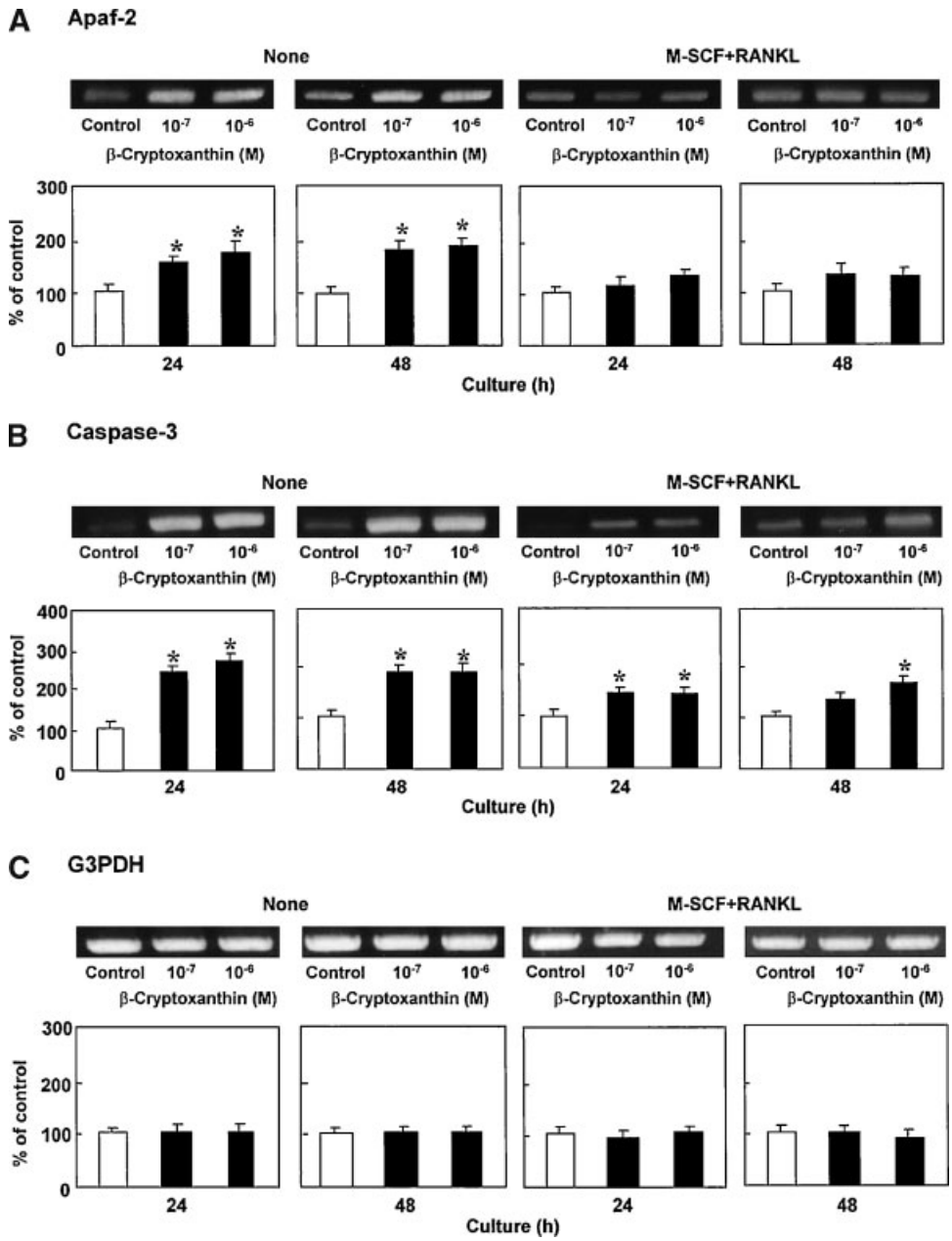

Fig. 6. Effect of β-cryptoxanthin on mRNA expression of Apaf-2 or caspase-3, which is related to stimulation of cell death and apoptosis, in osteoclastic cells formed in mouse marrow culture. Mouse marrow cells were cultured as described in the Materials and Methods. The adherent cells were cultured for 96 h in medium containing 0.5% FBS, M-CSF (10 ng/ml), and RANKL (50 ng/ml). After medium change, the osteoclast-like cells formed were cultured for 24 or 48 h in medium containing either vehicle (1% ethanol) or β-cryptoxanthin (10^{-7} or 10^{-6} M) in the absence or presence of M-CSF (10 ng/ml) plus RANKL (50 ng/ml). Total RNAs (4 μg) 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 ± SEM of five experiments). $*P < 0.01$ compared with the control (none) value.

sence or absence of M-CSF (10 ng/ml) and RANKL (50 ng/ml) for 24 or 48 h. TRACP activity was significantly increased in the presence of M-CSF (10 ng/ml) and RANKL (50 ng/ml) for 24 or 48 h as compared with the value obtained in the absence of M-CSF and RANKL. This increase was significantly inhibited with culture of β-cryptoxanthin (10^{-6} M).

The effect of β-cryptoxanthin on TRACP or cathepsin K mRNA expressions, which their proteins are related to bone-resorbing activity, in osteoclastic cells was examined (Fig. 9). The

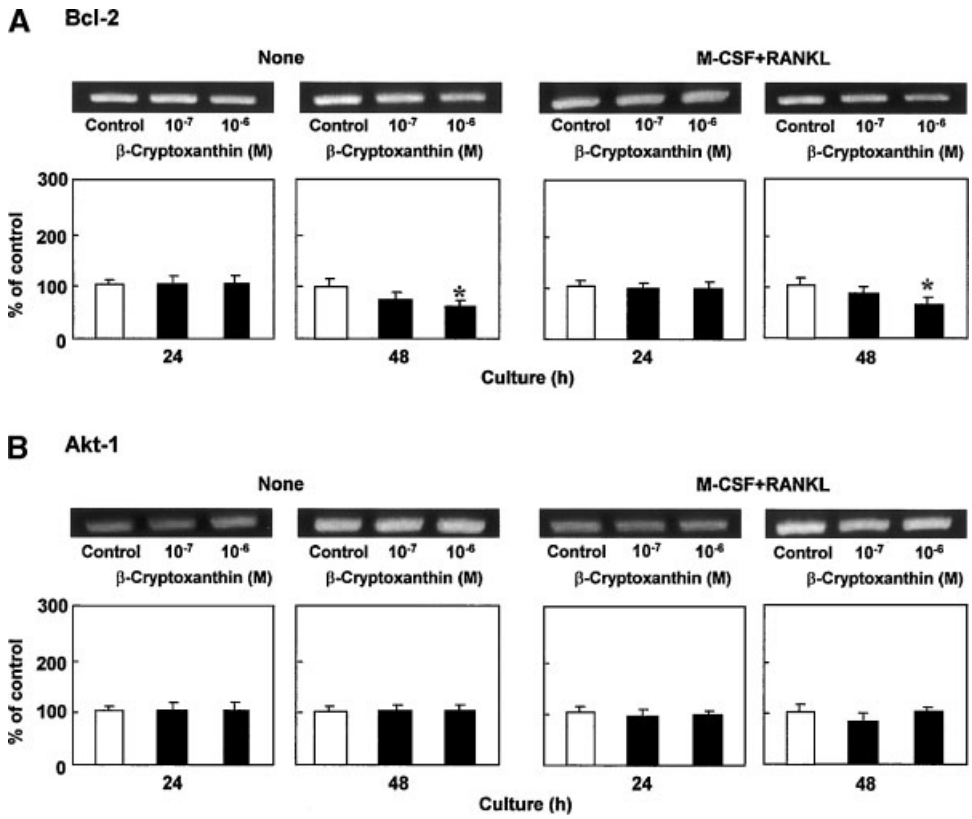

Fig. 7. Effect of β-cryptoxanthin on mRNA expression of Bcl-2 or Akt-1, which is related to rescue cell death and apoptosis, in osteoclastic cells formed in mouse marrow culture. Mouse marrow cells were cultured as described in the Materials and Methods. The adherent cells were cultured for 96 h in medium containing 0.5% FBS, M-CSF (10 ng/ml), and RANKL (50 ng/ml). After medium change, the osteoclast-like cells formed were cultured for 24 or 48 h in medium containing either vehicle (1% ethanol) or β-cryptoxanthin (10^{-7} or 10^{-6} M) in the absence or presence of M-CSF (10 ng/ml) plus RANKL (25 ng/ml). Total RNAs (4 μg) 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 ± SEM of five experiments). $*P < 0.01$ compared with the control (none) value.

expression of TRACP mRNA was significantly decreased with culture of β-cryptoxanthin (10^{-6} M) in the presence or absence of M-CSF (10 ng/ml) and RANKL (50 ng/ml) for 48 h (Fig. 9A). Cathepsin K mRNA expression was significantly decreased with culture of β-cryptoxanthin (10^{-6} M) in the presence or absence of M-CSF and RANKL for 24 or 48 h (Fig. 9B).

DISCUSSION

β-Cryptoxanthin has been shown to have stimulatory effects on bone formation and inhibitory effects on bone resorption in rat femoral tissues in vitro [Yamaguchi and Uchiyama, 2004]. β-Cryptoxanthin stimulates gene expression of proteins, which involve in osteoblastic bone formation, and that the carotenoid enhances cell proliferation and differentiation of osteoblastic MC3T3-E1 cells, supporting the view that the carotenoid can stimulate osteoblastic bone formation [Uchiyama and Yamaguchi, 2005b,c]. β-Cryptoxanthin has suppressive effects on RANKL-stimulated osteoclastogenesis from mouse marrow culture in vitro [Uchiyama and Yamaguchi, 2004]. This study, furthermore, was undertaken to determine whether β-cryptoxanthin has effect on osteoclasts formed in mouse marrow culture in vitro. We found that β-cryptoxanthin stimulates apoptotic cell death and its related gene expression in osteoclastic cells, suggesting that the carotenoid has inhibitory effects on mature osteoclasts.

The formation of osteoclastic cells reached to peak when M-CSF-dependent bone marrow macrophages were cultured in the presence M-CSF and RANKL for 4 days. Osteoclastogenesis was significantly inhibited in the presence of β-cryptoxanthin, supporting the view that the

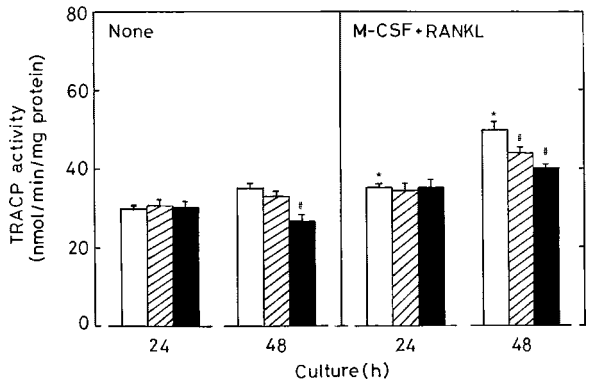

Fig. 8. Effect of β-cryptoxanthin on TRACP activity in osteoclastic cells. Mouse marrow cells were cultured as described in the Materials and Methods. The adherent cells were cultured for 96 h in medium containing 0.5% FBS, M-CSF (10 ng/ml), and RANKL (50 ng/ml). After medium change, the osteoclast-like cells formed were cultured for 24 or 48 h in medium containing either vehicle (1% ethanol) or β-cryptoxanthin (10^{-7} or 10^{-6} M) in the absence or presence of M-CSF (10 ng/ml) plus RANKL (50 ng/ml). The cell lysate of osteoclastic cells was used for assay of TRACP activity. Each value is the mean ± SEM of six cultures. $^{*}P < 0.01$ compared with the control (none) value obtained at culture for 24 or 48 h. White bars, control (none); hatched bars, with β-cryptoxanthin (10^{-7} M); black bars, with β-cryptoxanthin (10^{-6} M).

carotenoid has suppressive effects on osteoclast formation, as reported previously [Uchiyama and Yamaguchi, 2004]. After culture for 4 days with M-CSF and RANKL, the medium was changed to that with or without M-CSF plus RANKL, and the formed osteoclasts were further cultured for 24–72 h in the presence or absence of β-cryptoxanthin. Osteoclastic cells were gradually decreased when cultured without M-CSF and RANKL, indicating that cell death and apoptosis were induced. This decrease was significantly prevented in the presence of M-CSF and RANKL, suggesting that the cytokines act as survival factor. More recently, it has been reported that RANKL reduces the levels of Fas expression and Fas-mediated apoptosis, acting as a survival factor [Wu et al., 2005].

Culture with β-cryptoxanthin was found to have stimulatory effects on cell death and apoptosis of osteoclastic cells formed in mouse marrow culture. This effect was seen in the presence or absence of M-CSF and RANKL. β-Cryptoxanthin-induced decrease in osteoclastic cell number was significantly prevented in the presence of caspase-3 inhibitor. In addition, culture with β-cryptoxanthin caused DNA fragmentation in osteoclastic cells with or without M-CSF and RANKL. These observations indicate that β-cryptoxanthin has stimulatory effects on apoptotic cell death of osteoclastic cells in vitro.

The expression of caspase-3 mRNA or Apaf-2, which involves in apoptosis, in osteoclastic cells was found to stimulate with culture of β-cryptoxanthin in the absence of M-CSF and RANKL. β-Cryptoxanthin could stimulate caspase-3 mRNA expression in the presence of M-CSF and RANKL. This finding suggests that β-cryptoxanthin-induced apoptotic cell death is partly mediated through caspase-3 expression in osteoclastic cells. In addition, the expression of Bcl-2 mRNA, which involve in rescue of apoptosis, was significantly decreased with culture of β-cryptoxanthin in the presence or absence of M-CSF and RANKL. However, Akt-1 mRNA expression was not significantly changed with culture of β-cryptoxanthin. The decrease in Bcl-2 mRNA expression may partly contribute to the effect of β-cryptoxanthin in stimulating apoptotic cell death of osteoclastic cells.

Culture with β-cryptoxanthin was found to have suppressive effects on TRACP activity, TRACP, and cathepsin K mRNA expressions in osteoclastic cells in the presence or absence of M-CSF and RANKL. These findings suggest that β-cryptoxanthin can inhibit the enhancement of bone-resorbing activity in osteoclasts. β-Cryptoxanthin could inhibit various bone-resorbing factors-induced decrease in bone calcium content and increase in lactic acid production in rat femoral tissue culture system in vitro [Yamaguchi and Uchiyama, 2004]. Presumably, β-cryptoxanthin has inhibitory effects on activation of mature osteoclasts.

It has been reported that the serum concentration of β-cryptoxanthin increases due to consumption of vegetable juice in women to the range of 1.3×10^{-7} to 5.3×10^{-7} M [McEligot et al., 1999]. The anabolic effect of β-cryptoxanthin on bone calcification is observed at 10^{-7} or 10^{-6} M [Yamaguchi and Uchiyama, 2003]. The effect of β-cryptoxanthin in stimulating osteoblastic mineralization due to enhancing Runx2, α1(I) collagen, and alkaline phosphatase mRNA expressions in osteoblastic MC3T3-E1 cells is seen at 10^{-7} or 10^{-6} M of the carotenoid [Uchiyama and Yamaguchi, 2005c]. At this concentration, it is observed that β-cryptoxanthin inhibits oteoclastogenesis in mouse marrow culture in vitro [Uchiyama and

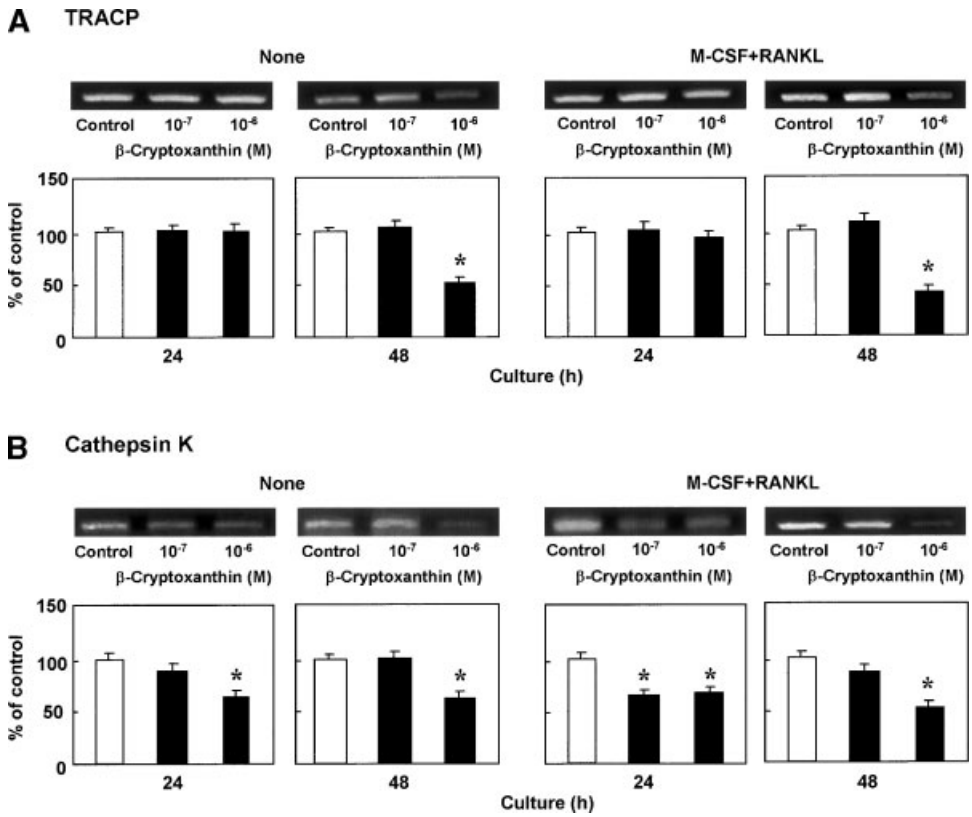

Fig. 9. Effect of β-cryptoxanthin on the expression of TRACP or cathepsin K mRNAs, which is related to osteoclastic bone resorption, in osteoclastic cells formed in mouse marrow culture. Mouse marrow cells were cultured as described in the Materials and Methods. The adherent cells were cultured for 96 h in medium containing 0.5% FBS, M-CSF (10 ng/ml), and RANKL (50 ng/ml). After medium change, the osteoclast-like cells formed were cultured for 24 or 48 h in medium containing either vehicle (1% ethanol) or β-cryptoxanthin (10^{-7} or 10^{-6} M) in the absence or presence of M-CSF (10 ng/ml) plus RANKL (50 ng/ml). Total RNAs (4 μg) 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 ± SEM of five experiments). $*P < 0.01$ compared with the control (none) value.

Yamaguchi, 2004], and that stimulates apoptotic cell death and suppresses TRACP activity and its related gene expression in osteoclastic cells in vitro. Thus β-cryptoxanthin has effects on both osteoblastic and osteoclastic cells in bone tissues with its physiologic levels in life style.

In conclusion, it has been demonstrated that β-cryptoxanthin, a kind of carotenoid, has stimulatory effects on apoptotic cell death due to acting gene expression of its related proteins, and that the carotenoid has suppressive effects on TRACP activity and gene expression of enzymes which involve in bone-resorbing activity in osteoclastic cells.

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