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β-Cryptoxanthin Stimulates Cell Differentiation and Mineralization in Osteoblastic MC3T3-E1 Cells

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Abstract The effect of β-cryptoxanthin, a kind of carotenoid, on cell differentiation and mineralization in osteoblastic MC3T3-E1 cells was investigated. Cells were cultured for 72 h in a minimum essential medium containing 10% fetal bovine serum (FBS), and the cells with subconfluency were changed to a medium containing either vehicle or β-cryptoxanthin (10^{-8} to 10^{-6} M) without FBS. Cells were cultured for 3 to 21 days. Gene expression in osteoblastic cells was determined using reverse transcription-polymerase chain reaction (RT-PCR). Culture with β-cryptoxanthin (10^{-7} or 10^{-6} M) for 3 days caused a significant increase in Runx2 type 1, Runx2 type 2, α1 (I) collagen, and alkaline phosphatase mRNA levels in osteoblastic cells. These increases were completely blocked in the presence of cycloheximide, an inhibitor of protein synthesis, or 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), an inhibitor of transcriptional activity. Meanwhile, vitamin A (10^{-6} M) did not have a significant effect on Runx2 type 1 mRNA expression in the cells. The effect of β-cryptoxanthin (10^{-6} M) in stimulating Runx2 type 1 and α1 (I) collagen mRNA levels, protein content, and alkaline phosphatase activity in the cells was also seen in the presence of vitamin A (10^{-6} M), suggesting that the mode of β-cryptoxanthin action differs from that of vitamin A. Prolonged culture with β-cryptoxanthin (10^{-6} M) for 3 to 21 days caused a significant increase in cell number, deoxyribonucleic acid (DNA) content, protein content, and alkaline phosphatase activity in osteoblastic cells, suggesting that β-cryptoxanthin stimulates cell proliferation and differentiation. Moreover, culture with β-cryptoxanthin (10^{-7} or 10^{-6} M) for 5 to 21 days caused a remarkable increase in mineralization. This study demonstrates that β-cryptoxanthin has a stimulatory effect on cell differentiation and mineralization due to enhancing gene expression of proteins, which involve in bone formation in osteoblastic MC3T3-E1 cells. J. Cell. Biochem. 95: 1224–1234, 2005. © 2005 Wiley-Liss, Inc.

Key words: β-cryptoxanthin; mineralization; Runx2; collagen; alkaline phosphatase; vitamin A; osteoblast

Bone loss with aging induces osteoporosis, which is widely recognized as a major public health problem. The decrease in bone mass may induce bone fracture. Bone loss may be due to decreased bone formation and increased bone resorption. Pharmacologic and nutritional factors are needed to prevent bone loss with increasing age [Bonjour et al., 1996]. The chemical compounds in food that act on bone metabolism, however, are poorly understood.

Carotenoids are present in fruit and vegetables. The effects of carotenoids on bone metabolism, however, have not yet been clarified. Recent studies have shown that β-cryptoxanthin has a unique anabolic effect on bone calcification in vitro [Yamaguchi and Uchiyama, 2003] and in vivo [Uchiyama et al., 2004a]. 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 a direct stimulatory effect on bone formation and an inhibitory effect on bone resorption in cultured bone tissues in vitro [Yamaguchi and Uchiyama, 2004]. The inhibitory action of β-cryptoxanthin on bone resorption may partly involve in a newly synthesized protein component that is related to receptor activator of NF-κB ligand (RANKL) stimulation in osteoclastogenesis [Uchiyama

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and Yamaguchi, 2004]. The action of β-cryptoxanthin on osteoblasts, however, has not been clarified yet.

The present study was undertaken to determine the effect of β-cryptoxanthin in osteoblastic MC3T3-E1 cells. We found that β-cryptoxanthin has a stimulatory effect on Runx2, α1(I) collagen, and alkaline phosphatase mRNA expression in osteoblastic cells, and that the prolonged culture with the carotenoid markedly enhances cell proliferation and differentiation, inducing osteoblastic mineralization in vitro.

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. Fetal bovine serum (FBS) was obtained from Bioproducts, Inc. Vitamin A (retinol), 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), cycloheximide, and Alizarin red S was purchased from Sigma (St. Louis, MO). β-Cryptoxanthin was obtained from Extrasynthese (Lyon-Nord, France). Other chemicals were of reagent grade and were obtained from Wako Pure Chemical Industries (Osaka, Japan). All water used were glass distilled.

Cell Culture

Osteoblastic MC3T3-E1 cells were cultured at 37°C in a CO_2 incubator in plastic dishes containing α-MEM supplemented with 10% FBS. They were subcultured every 3 days using 0.2% trypsin plus 0.02% EDTA in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS). For experiments, about 2.5×10^5 cells per dish were cultured for 72 h to obtain subconfluent monolayers in 35-mm plastic containing 2 ml α-MEM with 10% FBS. After the cells were rinsed with PBS, the medium was exchanged for medium without FBS containing either vehicle or β-cryptoxanthin (10^{-7} or 10^{-6} M) and the cells were cultured further for 1 to 21 days. 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 phosphate-buffer saline (PBS) solution at 100*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 Hemacytometer plate. For each dish, we took the average of two counts.

Determination of Specific mRNA by RT-PCR

Total RNAs were prepared as described previously [Chomczyshi and Sacchi, 1987]. Osteoblastic MC3T3-E1 cells with subconfluency were cultured for 24, 48, or 72 h in a medium containing either vehicle or β-cryptoxanthin (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 diethyl-pyrocarbonate-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 Runx2 type 1 cDNA were; 5′-ATGCGTATTCCTGTAGATCCGAG-3′ (sense strand, positions 1016–1038 of cDNA sequence) and 5′-CATCATTCCCGGCCATGACGGTAAC-3′ (antisense strand, positions 1451–1475) [Seth et al., 2000]. 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 Runx2 type 2 cDNA were; 5′-ATGCTTCATTCGCCTCACAAACAACC-3′ (sense strand, positions 1–26 of cDNA sequence) and 5′-TGGTGCGGTTGTCGTGCGGC-3′ (antisense strand, positions 513–533) [Seth et al., 2000]. The pair of oligonucleotide primers was designed to amplify a 528-bp sequence from the mRNA of mouse Runx2 type 2. Primers for amplification of mouse α1(I) collagen cDNA were: 5′-TTCTCCTGGTAAAGATGGTGC-3′ (sense strand, positions 2232–2252 of cDNA sequence) and 5′-GGACCAGCATCACCTTTAACA-3′ (antisense strand, positions 2466–2486) [Luppen et al., 2003]. The pair of oligo-

nucleotide primers was designed to amplify a 254 bp sequence from the mRNA of α1(I) collagen. Primers for amplification of mouse alkaline phosphatase cDNA were: 5′-GATCGGGACTGGTACTCGGATAA-3′ (sense strand, positions 729–751 of cDNA sequence) and 5′-CACATCAGTTCTGTTCTTCGGGTAC-3′ (antisense strand, positions 860–884) [Seth et al., 2000]. The pair of oligonucleotide primers was designed to amplify a 155-bp sequence from the mRNA of alkaline phosphatase. For semiquantitative PCR, glyceroaldehyde-3-phosphate dehydrogenase (G3PDH) was used as an internal control to evaluate total RNA input. Primers for 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 reaction mixture (20 μl) containing 2 or 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 30 cycles under the following conditions: denaturation for 30 s 94°C, annealing for 30 s at 56°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 FluoroImager SI (Amersham Pharmacia Biotech).

Biochemical Analysis

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 [Flanagan and Nichols, 1962]. After alkali extraction, the samples were centrifuged at 10,000*g* for 5 min, and the supernatant was collected. DNA content in the supernatant was determined by the method of Ceriotti [1955] and expressed as the amount of DNA (μg) per dish.

To determine the protein concentration in osteoblastic cells, the cells were washed three times with PBS, scraped into 0.5 ml of ice-cold 0.25-M sucrose solution, and disrupted for 30 s with an ultrasonic device. Protein concentration in the cell homogenate was determined by the method of Lowry et al. [1951] and expressed as the amount of protein (μg) per dish.

To assay alkaline phosphatase activity in the cells after appropriate treatment periods, the cells were washed three times with PBS, scraped into 0.5 ml of ice-cold 0.25 M sucrose solution, and disrupted for 30 s with an ultrasonic device. The supernatant, centrifuged at 600*g* for 5 min, was used to measure enzyme activity. The enzyme assay described below was carried out under optimal conditions. Alkaline phosphatase activity was determined by the method of Walter and Schutt [1965]. The enzyme activity was expressed as nanomoles of p-nitrophenol liberated per minute per milligram of protein.

Alizarin Red Staining

Osteoblastic MC3T3-E1 cells (2.5×10^5 cells) were cultured for 72 h in a α-MEM containing 10% FBS. Cells with subconfluency were changed to a Dullbecco's modified essential medium (DMEM) containing ascorbic acid (100 μg/ml) and 4 mMβ-glycerophosphate in the presence or absence of β-cryptoxanthin (10^{-7} or 10^{-6} M) with 10%FBS. After medium change, cells were cultured for 5, 10, 15, or 21 days. The medium was changed with every 3 days. At each time point, cells were rinsed with PBS, and fixed on ice with 70% ethanol for 15 min for alizarin red staining of calcium [Kamiya et al., 2002]. The Alizarin red solution (40 mM, pH 4.2) was filtered through Whatman paper and applied to the fixed wells for 30 min at room temperature. Nonspecific staining was removed by several washed in water.

Statistical Analysis

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

RESULTS

Effect of β-Cryptoxanthin on Gene Expression in Osteoblastic Cells

Osteoblastic MC3T3-E1 cells were cultured for 72 h in a medium containing 10% FBS. Cells with subuconfluency were changed to a medium containing either vehicle or β-cryptoxanthin (10^{-6} M). After medium change, cells were

cultured for 24, 48, or 72 h. The alteration in Runx2 type 1, α1(I) collagen, or alkaline phosphatase mRNA expression was examined (Fig. 1). Culture with β-cryptoxanthin for 24, 48, or 72 h caused a significant increase in Runx2 type 1 and alkaline phosphatase mRNA levels in the cells. The expression of α1(I) collagen mRNA was significantly increased by culture with β-cryptoxanthin for 48 or 72 h. Meanwhile, G3PDH mRNA level was not appreciably changed in the presence of β-cryptoxanthin.

The effect of β-cryptoxanthin with increasing concentrations on Runx2 type 1, Runx2 type 2, α1(I) collagen, or alkaline phosphatase mRNAs expression was examined (Fig. 2). Cells with subconfluency were cultured for 72 h in the presence of β-cryptoxanthin (10^{-8}–10^{-6} M) without FBS. The expression of Runx2 type 1, Runx2 type 2, α1(I) collagen, or alkaline phosphatase mRNAs was significantly increased in the presence of β-cryptoxanthin (10^{-7} or 10^{-6} M). Runx2 type 2 or α1(I) collagen mRNA levels were significantly raised by β-cryptoxanthin of 10^{-8}–10^{-6} M.

The effect of cycloheximide (Fig. 3) or DRB (Fig. 4) on the β-cryptoxanthin-increased Runx2 type 1, Runx2 type 2, α1(I) collagen, or alkaline phosphatase mRNAs expression was examined. Cells with subconfluency were cultured in a medium containing either vehicle or β-cryptoxanthin (10^{-6} M) in the presence or absence of cycloheximide (10^{-7} M) or DRB (10^{-6} M) without FBS for 72 h. The expression of Runx2 type 1, Runx2 type 2, α1(I) collagen, or alkaline phosphatase mRNAs increased by β-cryptoxanthin (10^{-6} M) was completely pre-

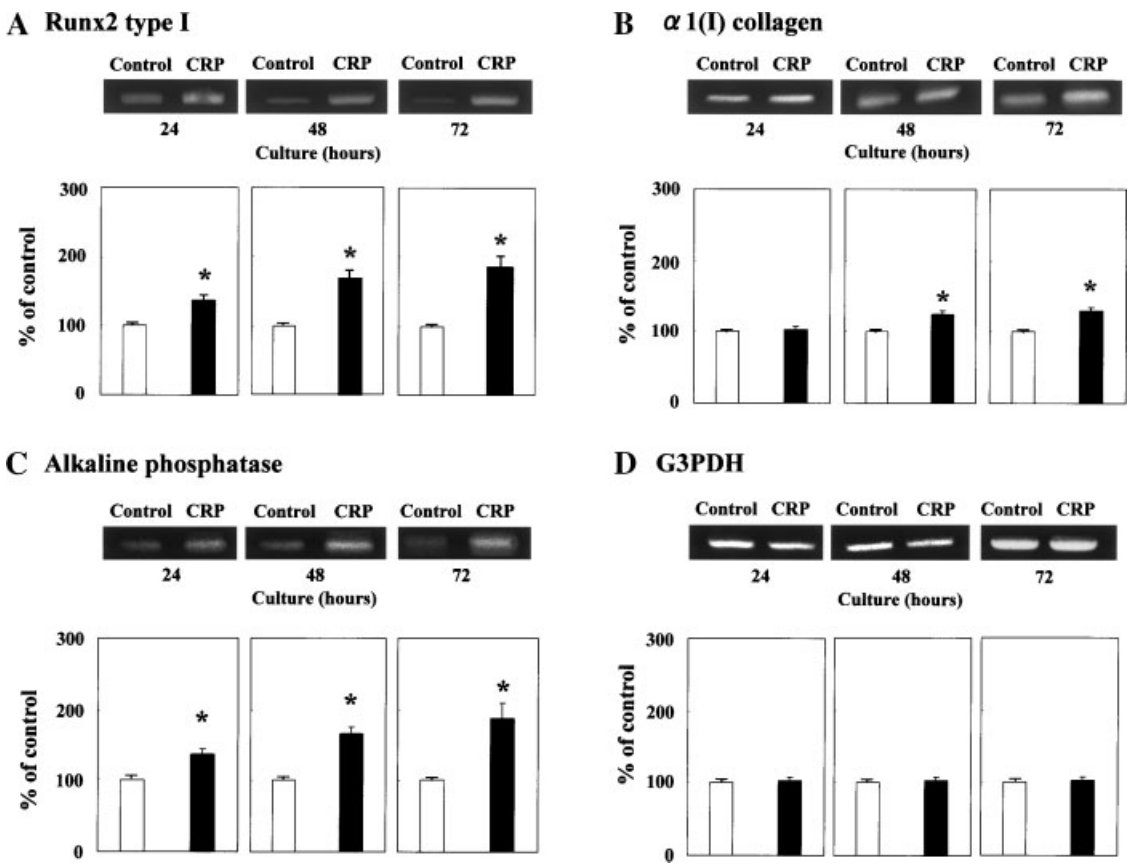

Fig. 1. Effect of β-cryptoxanthin (CRP)on the expression of Runx2 type 1 (**A**), α1(I) collagen (**B**), alkaline phosphatase (**C**), or G3PDH (**D**) mRNAs in osteoblastic MC3T3-E1 cells. Osteoblastic cells (2.5×10^5 cells) were cultured for 72 h in a medium containing 10% FBS. Cells with subconfluency were changed to a medium without FBS in the presence or absence of CRP (10^{-6} M). After medium change, cells were cultured for 24, 48, or 72 h. Total RNAs (4 μg for Runx2 type 1 mRNA and 2 μg for α1(I) collagen, alkaline phosphatase, or G3PDH mRNAs) extracted from the 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 in the cells cultured for 24, 48, or 72 h in the presence of CRP were indicated as % of control (mean ± SEM for five experiments). $^*P < 0.01$, compared with the control value.

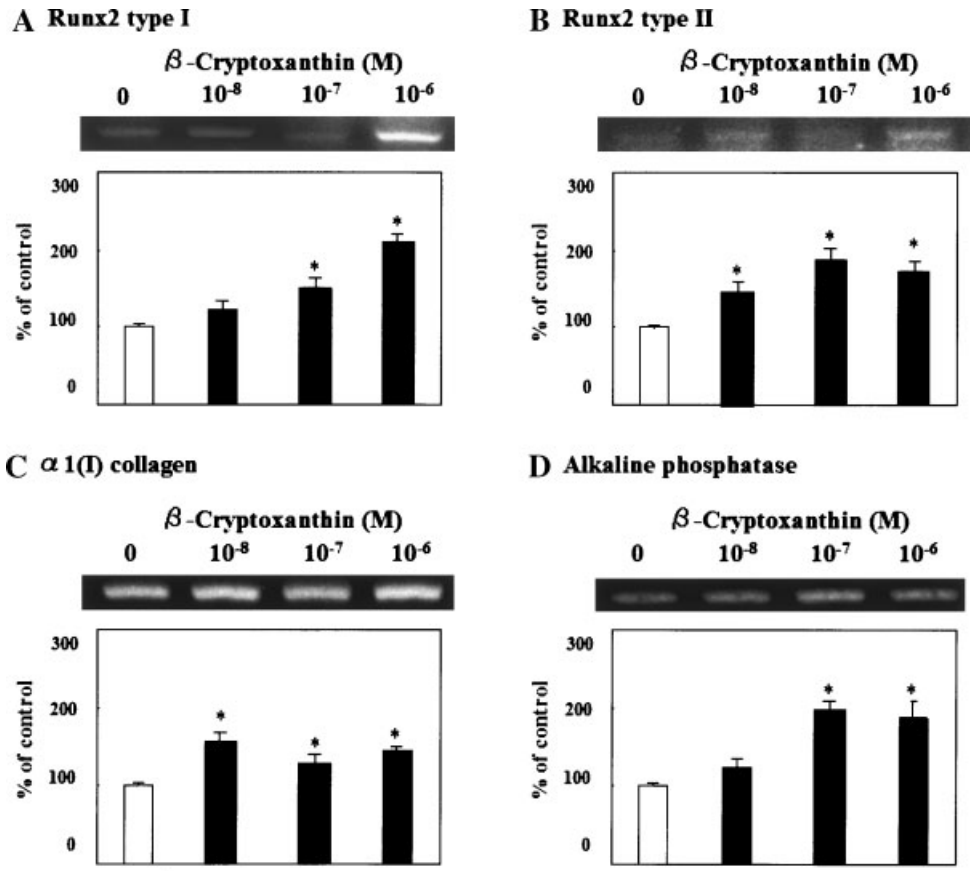

Fig. 2. Effect of β-cryptoxanthin with increasing concentrations on the expression of Runx2 type 1 (**A**), Runx2 type 2 (**B**), α1(I) collagen (**C**), or alkaline phosphatase (**D**) mRNAs in osteoblastic MC3T3-E1 cells. Osteoblastic cells (2.5×10^5 cells) were cultured for 72 h in a medium containing 10% FBS. Cells with subconfluency were changed to a medium without FBS in the presence or absence of β-cryptoxanthin (CRP; 10^{-8}–10^{-6} M). After medium change, cells were cultured for 72 h. Total RNAs (4 μg for Runx2 type 1 and type 2 mRNAs and 2 μg for α1(I) collagen, alkaline phosphatase mRNAs) extracted from the 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 in the cells cultured for 72 h in the presence of CRP were indicated as % of control (mean ± SEM for five experiments). $^*P < 0.01$, compared with the control value.

vented in the presence of cycloheximide or DRB. G3PDH mRNA levels were not significantly changed in the presence of cycloheximide (10^{-7} M) or DRB (10^{-6} M) (data not shown).

The effect of β-cryptoxanthin on Runx2 type 1 and α1(I) collagen mRNA expressions was examined in the presence of vitamin A (Fig. 5). Osteoblastic cells were cultured for 24–72 h in a medium containing either vehicle, β-cryptoxanthin (10^{-6} M), vitamin A (10^{-6} M), or β-cryptoxanthin (10^{-6} M) plus vitamin A (10^{-6} M). Vitamin A significantly increased α1(I) collagen level in osteoblastic cells, while it did not cause a significant alteration in Runx2 type 1 mRNA level. The effect of β-cryptoxanthin in stimulating Runx2 type 1 and α1(I) collagen mRNAs expression was also seen in the presence of vitamin A.

When osteoblastic cells were cultured for 72 h in the presence of β-cryptoxanthin (10^{-7} or 10^{-6} M), the protein content or alkaline phosphatase activity in the cells was significantly raised (Table I). Meanwhile, vitamin A (10^{-7} or 10^{-6} M) did not cause a significant increase in protein content in the cells. Alkaline phosphatase activity in the cells was significantly raised by culture with vitamin A (10^{-7} or 10^{-6} M). The effect of β-cryptoxanthin (10^{-7} or 10^{-6} M) in increasing protein content and alkaline phosphatase activity in the cells was also seen in the presence of vitamin A (10^{-6} M).

Thus, the mode of action of β-cryptoxanthin in stimulating transcriptional activity may differ from that of vitamin A, which is mediated through retinoid X receptors (RXR) in the nucleus of osteoblastic cells.

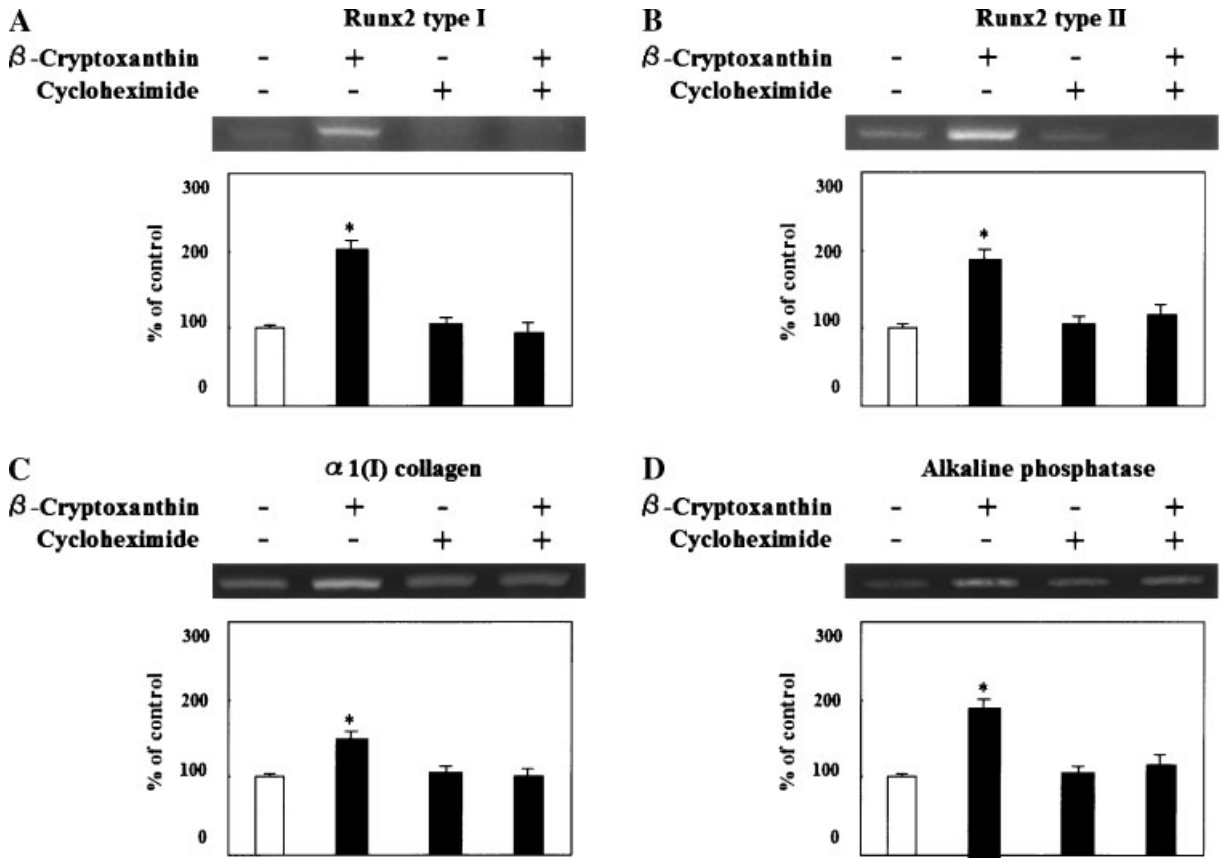

Fig. 3. Effect of β-cryptoxanthin on the expression of Runx2 type 1 (**A**), Runx2 type 2 (**B**), α1(I) collagen (**C**), or alkaline phosphatase (**D**) mRNAs in osteoblastic MC3T3-E1 cells in the presence of cycloheximide. Osteoblastic cells (2.5×10^5 cells) were cultured for 72 h in a medium containing 10% FBS. Cells with subconfluency were changed to a medium without FBS in the presence or absence of β-cryptoxanthin (CRP; 10^{-6} M) or cycloheximide (l0^{-7} M). After medium change, cells were cultured for 72 h. Total RNAs (4 μg for Runx2 type 1 and type 2 mRNAs and 2 μg for α1(I) collagen or alkaline phosphatase mRNAs) extracted from the 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 ± SEM for five experiments). *$P < 0.01$, compared with the control value.

Effect of Prolonged Culture With β-Cryptoxanthin in Osteoblastic Cells

Osteoblastic MC3T3-E1 cells with subconfluency were cultured for 3, 6, 9, 12, or 21 days in a medium containing either vehicle or β-cryptoxanthin (10^{-7} or 10^{-6} M), and the change in cellular component was examined. The number of osteoblastic cells was progressively increased by prolonged culture with β-cryptoxanthin (10^{-7} or 10^{-6} M) (Fig. 6), indicating that the carotenoid stimulates cell proliferation. Cellular DNA content was progressively increased by culture with β-cryptoxanthin (10^{-7} or 10^{-6} M) (Fig. 7). Protein content in the cells was significantly increased by culture with β-cryptoxanthin (10^{-6} M) for 3 to 21 days (Fig. 8). Alkaline phosphatase activity in osteoblastic cells was progressively increased with prolonged culture without carotenoid (Fig. 9), indicating that the cells are differentiated with culture. This increase was significantly enhanced in the presence of β-cryptoxanthin (10^{-6} M) (Fig. 9).

Effect of β-Cryptoxanthin on Mineralization in Osteoblastic Cells

Osteoblastic cell with subconfluency were cultured for 5, 10, 15, or 21 days in a medium containing either vehicle or β-cryptoxanthin (10^{-7} or 10^{-6} M), and the mineralization was examined (Fig. 10). The results with Alizarin red staining for calcium showed that mineralization was significantly stimulated in the presence of β-cryptoxanthin (10^{-7} or 10^{-6} M). The enhancement of mineralization was observed from 5 days of culture. The effect was

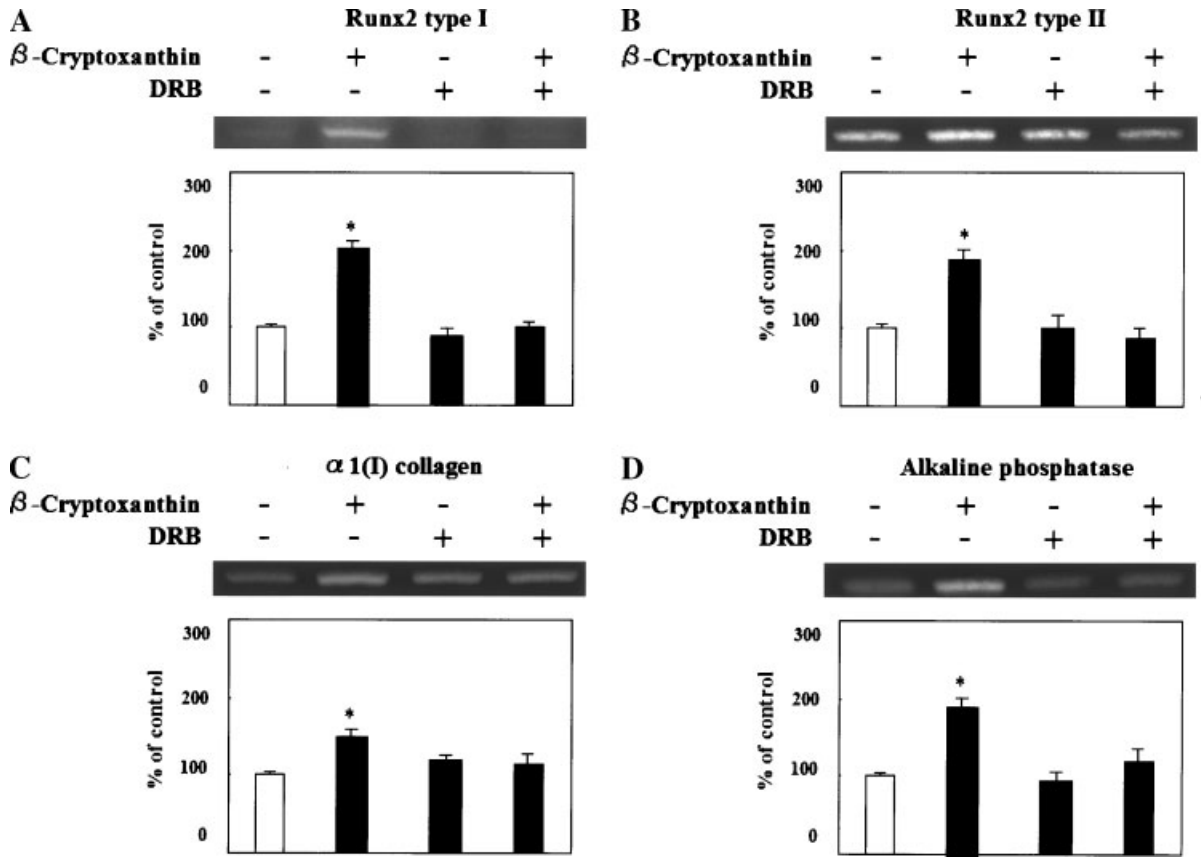

Fig. 4. Effect of β-cryptoxanthin on the expression of Runx2 type 1 (**A**), Runx2 type 2 (**B**), α1(I) collagen (**C**), or alkaline phosphatase (**D**) mRNAs in osteoblastic MC3T3-E1 cells in the presence of DRB. Osteoblastic cells (2.5×10^5 cells) were cultured for 72 h in a medium containing 10% FBS. Cells with subconfluency were changed to a medium without FBS in the presence or absence of β-cryptoxanthin (CRP; 10^{-6} M) or DRB (10^{-6} M). After medium change, cells were cultured for 72 h. Total RNAs (4 μg for Runx2 type 1 and type 2 mRNAs and 2 μg for α1(I) collagen or alkaline phosphatase mRNAs) extracted from the 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 ± SEM for five experiments). $^*P < 0.01$, compared with the control value.

markedly enhanced by 21-day culture with β-cryptoxanthin (10^{-7} or 10^{-6} M).

DISCUSSION

β-Cryptoxanthin has been shown to have a stimulatory effect on bone formation and an inhibitory effect on bone resorption using rat femoral tissues in vitro [Yamaguchi and Uchiyama, 2004]. β-Cryptoxanthin has a suppressive effect on RANKL-stimulated osteoclastogenesis from mouse marrow culture in vitro [Uchiyama and Yamaguchi, 2004]. The present study, moreover, was undertaken to determine whether β-cryptoxanthin has an effect on osteoblastic function in vitro. We found that 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, inducing mineralization in the cells. This finding may support the view that β-cryptoxanthin can stimulate osteoblastic bone formation.

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} and 10^{-6} M in vitro [Yamaguchi and Uchiyama, 2003]. β-Cryptoxanthin (10^{-7} or 10^{-6} M) has been found to increase the expression of Runx2, α1(I) collagen and alkaline phosphatase mRNA in osteoblastic MC3T3-E1 cells. Runx2 (Cbfa1) is a member of the runt domain family of transcription factors, and it is involved in bone development [Komori et al., 1997]. α1(I)

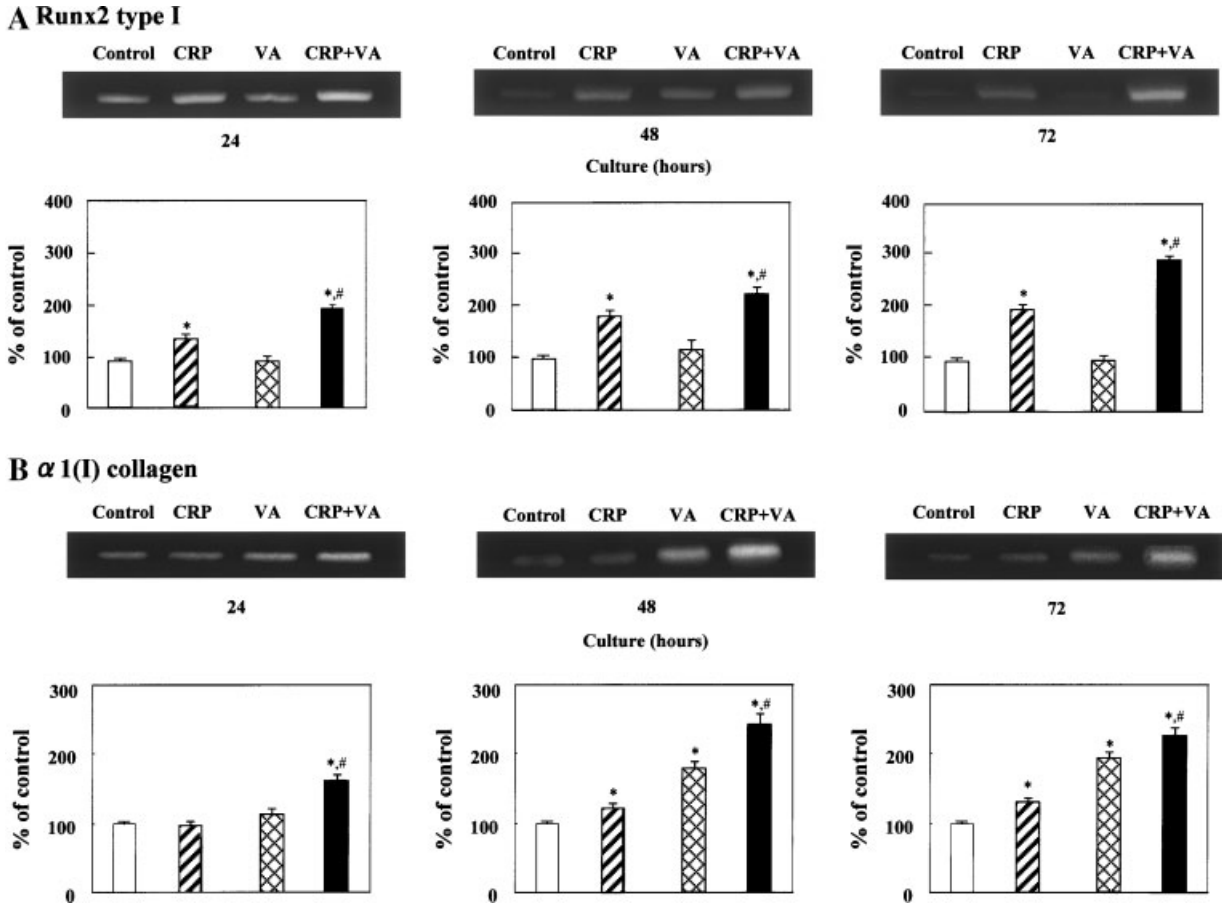

Fig. 5. Effect of β-cryptoxanthin (CRP) and vitamin A on the expression of Runx2 type 1 (**A**) or α1(I) collagen (**B**) mRNAs in osteoblastic MC3T3-E1 cells. Osteoblastic cells (2.5×10^5 cells) were cultured for 72 h in a medium containing 10% FBS. Cells with subconfluency were changed to a medium containing either vehicle, CRP (10^{-6} M), vitamin A (VA; 10^{-6} M) or CRP (10^{-6} M) plus VA (10^{-6} M) without FBS. After medium change, cells were cultured for 24, 48, or 72 h. Total RNAs (4 μg for Runx2 type 1 mRNA and 2 μg for α1(I) collagen) extracted from the 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 with the control (mean ± SEM for five experiments). $^{*}P < 0.01$, compared with the control value. $^{\#}P < 0.01$, compared with the value obtained from CRP alone.

TABLE I. Interaction of β-Cryptoxanthin and Vitamin A on Protein Concentration and Alkaline Phosphatase Activity in Osteoblastic MC3T3-E1 Cells

Treatment	Protein (μg/dish)	Alkaline phosphatase (nmol/min/mg protein)
Control	89.9 ± 3.38	13.1 ± 0.42
β-Cryptoxanthin (10^{-7} M)	99.6 ± 2.80*	16.4 ± 1.52*
β-Cryptoxanthin (10^{-6} M)	109.3 ± 3.17*	18.6 ± 1.29*
Vitamin A (10^{-7} M)	93.3 ± 1.76	19.2 ± 1.22**
Vitamin A (10^{-6} M)	93.6 ± 2.92	21.9 ± 0.98**
β-Cryptoxanthin (10^{-7} M) + vitamin A (10^{-6} M)	100.2 ± 0.75**	32.0 ± 0.94**,***
β-Cryptoxanthin (10^{-6} M) + vitamin A (10^{-6} M)	119.4 ± 2.07**,***	24.3 ± 1.54**,***

Cells were cultured for 72 h in a medium containing 10% FBS, and then changed to medium containing either vehicle, β-cryptoxanthin (10^{-7} or 10^{-6} M), β-cryptoxanthin (10^{-7} M) plus vitamin A (10^{-6} M) or β-cryptoxanthin (10^{-6} M) plus vitamin A (10^{-6} M). After medium change, cells were cultured for 72 h. Each value is the mean ± SEM of five experiments with separate culture.
$^{*}P < 0.05$, compared with the control value.
$^{**}P < 0.01$, compared with the control value.
$^{***}P < 0.01$, compared with the value obtained from β-cryptoxanthin alone.

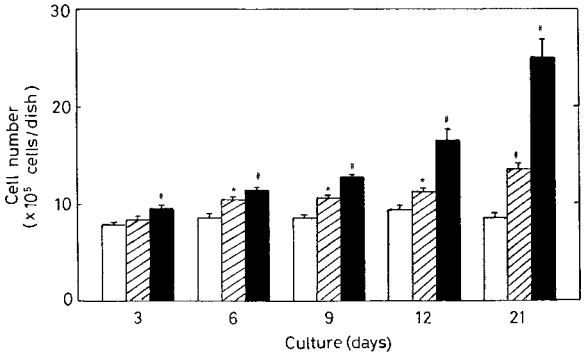

Fig. 6. Effect of β-cryptoxanthin on cell proliferation of osteoblastic MC3T3-E1 cells. Osteoblastic cells (2.5×10^5 cells) were cultured for 72 h in a medium containing 10% FBS. Cells with subconfluency were changed to a medium without FBS in the presence or absence of CRP (10^{-7} or 10^{-6} M). After medium change, cells were cultured 3, 6, 9, 12, or 21 days. After trypsinization of the cells in each cultured dish, cells were collected and counted. Each value in the mean ± SEM of six culture. $^*P < 0.05$ or $^{\#}P < 0.01$, compared with the control (none) value. White bars, control; hatched bars, β-cryptoxanthin (10^{-7} M); black bars, β-cryptoxanthin (10^{-6} M).

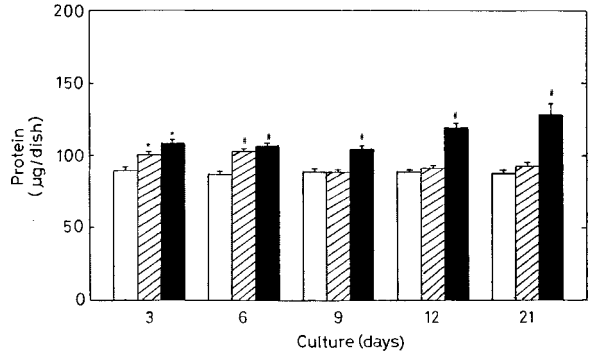

Fig. 8. Effect of β-cryptoxanthin on protein content in osteoblastic MC3T3-E1 cells. Osteoblastic cells (2.5×10^5 cells) were cultured for 72 h in a medium containing 10% FBS. Cells with subconfluency were changed to a medium without FBS in the presence or absence of CRP (10^{-7} or 10^{-6} M). After medium change, cells were cultured 3, 6, 9, 12, or 21 days. Cells were washed with PBS and scraped to assay protein content. Each value in the mean ± SEM of six culture. $^*P < 0.05$ or $^{\#}P < 0.01$, compared with the control value. White bars, control; hatched bars, β-cryptoxanthin (10^{-7} M); black bars, β-cryptoxanthin (10^{-6} M).

collagen is a matrix protein that is related to bone formation and mineralization in osteoblast lineage cells [Lian et al., 1999]. Alkaline phosphatase participates in mineralization process in osteoblastic cells [Yohay et al., 1994; Lian et al., 1999]. β-Cryptoxanthin has a stimulatory effect on the expression of gene for proteins that involve in osteoblastic bone formation.

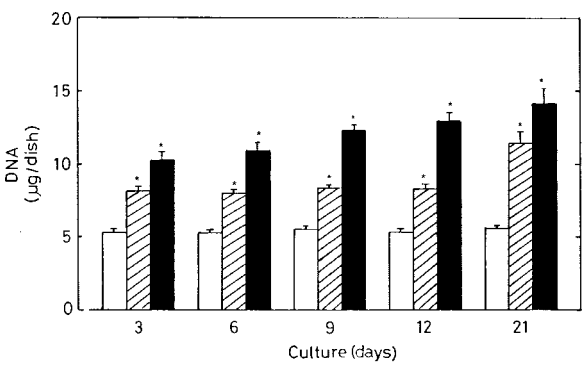

Fig. 7. Effect of β-cryptoxanthin on DNA content in osteoblastic MC3T3-E1 cells. Osteoblastic cells (2.5×10^5 cells) were cultured for 72 h in a medium containing 10% FBS. Cells with subconfluency were changed to a medium without FBS in the presence or absence of CRP (10^{-7} or 10^{-6} M). After medium change, cells were cultured 3, 6, 9, 12, or 21 days. Cells were washed FBS and scraped to assay DNA content. Each value in the mean ± SEM of six culture. $^*P < 0.01$, compared with the control value. White bars, control (none); hatched bars, β-cryptoxanthin (10^{-7} M); black bars, β-cryptoxanthin (10^{-6} M).

The effect of β-cryptoxanthin in stimulating Runx2, α1(I) collagen, and alkaline phosphatase mRNA expression in osteoblastic MC3T3-E1 cells was found to prevent completely in the presence of cycloheximide, an inhibitor of protein synthesis, or DRB, an inhibitor of transcriptional activity. This result suggests that β-cryptoxanthin stimulates transcriptional activity in osteoblastic MC3T3-E1 cells.

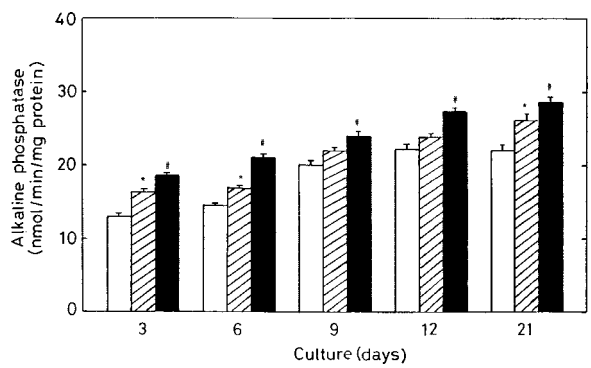

Fig. 9. Effect of β-cryptoxanthin on alkaline phosphatase activity in osteoblastic MC3T3-E1 cells. Osteoblastic cells (2.5×10^5 cells) were cultured for 72 h in a medium containing 10% FBS. Cells with subconfluency were changed to a medium without FBS in the presence or absence of β-cryptoxanthin (10^{-7} or 10^{-6} M). After medium change, cells were cultured 5, 10, 15, or 21 days. Cells were washed with PBS and scraped to assay the enzyme activity. Each value in the mean ± SEM of six culture. $^*P < 0.05$ or $^{\#}P < 0.01$, compared with the control value. White bars, control; hatched bars, β-cryptoxanthin (10^{-7} M); black bars, β-cryptoxanthin (10^{-6} M).

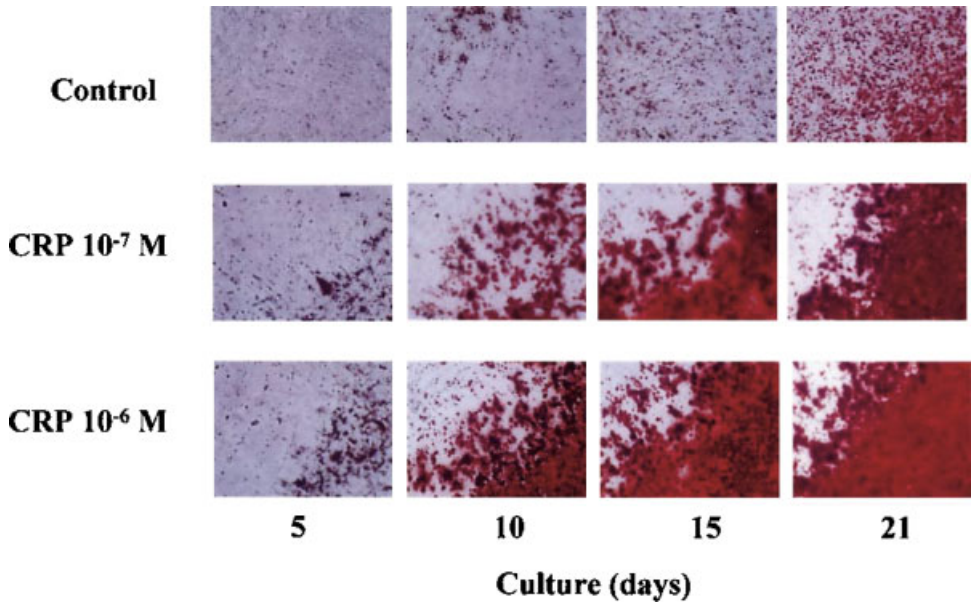

Fig. 10. Effect of β-cryptoxanthin on mineralization in osteoblastic MC3T3-E1 cells. Osteoblastic cells (2.5×10^5 cells) were cultured for 72 h in a medium containing 10% FBS. Cells with subconfluency were changed to a medium with 10% FBS in the presence or absence of β-cryptoxanthin (10^{-7} or 10^{-6} M). After medium change, cells were cultured 5, 10, 15, or 21 days. Cells were washed with PBS and stained Alizarin red staining. The figure shows one of five experiments with separate culture. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Vitamin A has been shown to stimulate of mouse osteoblastic cells [Park et al., 1997]. Retinol and β-carotene inhibit the proliferation of osteoblastic MC3T3-E1 cells as well as DNA synthesis of the cells, and retinol induces differentiation of the MC3T3-E1 cells, by increasing alkaline phosphatase activity dose dependently (10^{-9} to 10^{-7} M) [Park et al., 1997]. We confirmed that vitamin A (10^{-7} or 10^{-6} M) increases alkaline phosphatase activity in osteoblastic cells. β-Cryptoxanthin (10^{-7} or 10^{-6} M) caused a significant increase in alkaline phosphatase activity and protein content in osteoblastic cells. Such effect of β-cryptoxanthin was also seen in the presence of vitamin A (10^{-6} M). Moreover, the stimulatory effect of β-cryptoxanthin on the expression of Runx2 type 1 and α1(I) collagen mRNAs was also observed in the presence of vitamin A. Vitamin A did not have a siginificant effect on Runx2 type 1 mRNA expression in osteoblastic MC3T3-E1 cells. Thus, the mode of action of β-cryptoxanthin on gene expression in osteoblastic cells may differ from that of vitamin A that is mediated through RXR receptor in the nucleus of the cells [MacDonald et al., 1993]. It is speculated that β-cryptoxanthin may be able to bind other receptors (including orphan receptors), and that the carotenoid may stimulate transcriptional activity in osteoblastic cells. This remains to be elucidated, however.

β-Cryptoxanthin has been found to have a stimulatory effect on cell number, DNA content, protein content and alkaline phosphatase activity in osteoblastic MC3T3-E1 cells, when the cells were cultured for 3 to 21 days. Prolonged culture with β-cryptoxanthin has an anabolic effect on osteoblastic function. β-Cryptoxanthin may stimulate proliferation and differentiation of osteoblastic cells.

The mineralization by osteoblastic cells was induced by the prolonged culture with β-cryptoxanthin for 21 days. The stimulatory effect of β-cryptoxanthin on mineralization may result from the carotenoid-induced proliferation and differentiation of osteoblastic cells. It may be important; moreover, that β-cryptoxanthin stimulates gene expression for proteins that involve in bone formation and mineralization by osteoblastic cells.

β-Cryptoxanthin can stimulate bone formation and calcification in bone tissues in vitro [Yamaguchi and Uchiyama, 2003, 2004], and the oral administration has an anabolic effect on bone components in the femoral-diaphyseal and -metaphyseal tissues of male and female rats in vivo [Uchiyama et al., 2004a,b]. The intake of β-cryptoxanthin may have a preventive effect

on bone loss with increasing age. The present finding that β-cryptoxanthin promotes osteoblastic mineralization due to enhancing gene expression may support the view that the carotenoid has an important role as a stimulatory factor on bone formation.

In conclusion, it has been demonstrated that β-cryptoxanthin has a stimulatory effect on gene expression for proteins that involve in osteoblastic bone formation and mineralization in osteoblastic MC3T3-E1 cells.

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