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Biochemical Pharmacology 67 (2004) 1297–1305
Biochemical Pharmacology
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Inhibitory effect of β-cryptoxanthin on osteoclast-like cell formation in mouse marrow cultures

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Received 5 September 2003; accepted 14 November 2003

Abstract

The carotenoid β-cryptoxanthin has been shown to have an inhibitory effect on bone-resorping factor-stimulated bone resorption in rat bone tissues *in vitro*. The effect of β-cryptoxanthin on osteoclast-like cell formation in mouse marrow culture *in vitro* was investigated. The bone marrow cells were cultured for 7 days in α-minimal essential medium containing a bone-resorbing agent [parathyroid hormone (1–34) (PTH), prostaglandin E_2, 1,25-dihydroxyvitamin D_3, lipopolysaccharide, or tumor necrosis factor-α (TNFα)] with an effective concentration. Osteoclast-like cell formation was estimated by staining for tartrate-resistant acid phosphatase, a marker enzyme of osteoclasts. The presence of PTH (10^{-7} M), prostaglandin E_2 (10^{-5} M), 1,25-dihydroxyvitamin D_3 (10^{-7} M), lipopolysaccharide (10 μg/mL), or TNFα (10 ng/mL) induced a remarkable increase in osteoclast-like multinucleated cells. These increases were significantly inhibited in the presence of β-cryptoxanthin (10^{-8} to 10^{-6} M). β-Cryptoxanthin (10^{-7} and 10^{-6} M) significantly inhibited dibutyryl cyclic adenosine monophosphate (DcAMP) (10^{-5} M) or phorbol 12-myristate 13-acetate (PMA) (10^{-5} M), an activator of protein kinase C, induced osteoclast-like cell formation. Also, β-cryptoxanthin (10^{-7} and 10^{-6} M) had a significant inhibitory effect on osteoclast-like formation induced by receptor activator of NF-κB ligand (RANKL) (10 and 20 ng/mL) in the presence of macrophage colony-stimulating factor (M-CSF) (10 and 20 ng/mL). The stimulatory effect of RANKL and M-CSF on osteoclast-like cell formation was significantly enhanced in the presence of PMA, while such an effect was not seen by DcAMP. β-Cryptoxanthin (10^{-6} M) significantly inhibited osteoclast-like cell formation induced by RANKL and M-CSF in the presence of PMA or DcAMP. Moreover, the inhibitory effect of β-cryptoxanthin on RANKL plus M-CSF-, PTH-, or TNFα-induced osteoclast-like cell formation was not observed in the presence of cycloheximide (10^{-7} M), an inhibitor of protein synthesis at translational process, or 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (10^{-6} M), an inhibitor of transcription. This study demonstrates that β-cryptoxanthin has a potent inhibitory effect on osteoclast-like cell formation in mouse marrow culture. The inhibitory action of β-cryptoxanthin may partly involve in a newly synthesized protein component which is related to RANKL stimulation in osteoclastogenesis.

Keywords: β-Cryptoxanthin; Osteoclast differentiation; RANKL; Bone resorption

1. Introduction

Aging induces a decrease in bone mass [1,2]. Osteoporosis with its accompanying decrease in bone mass is widely recognized as a major public health problem. The most dramatic expression of the disease is represented by fractures of the proximal femurs [3,4]. Bone loss with increasing age may be due to decreased bone formation and increased bone resorption. Pharmacological and nutritional factors may prevent bone loss with increasing age [5]. The chemical compounds in food that act on bone metabolism, however, are poorly understood.

The anticarcinogenic effects of various micronutrients and phytochemicals found in vegetables and fruits, such as carotenoids, have been demonstrated in laboratory studies [6]. Carotenoids are present in fruit and vegetables. Carotenoids have been shown to play a possible biological role

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Abbreviations: α-MEM, α-minimal essential medium; PTH, parathyroid hormone (1–34); PGE_2, prostaglandin E_2; VD_3, 1,25-dihydroxyvitamin D_3; LPS, lipopolysaccharide; TNFα, tumor necrosis factor-α; TRACP, tartrate-resistant acid phosphatase; MNCs, multinucleated cells; PMA, phorbol 12-myristate 13-acetate; DcAMP, dibutyryl cyclic adenosine monophosphate; M-CSF, macrophage colony-stimulating factor; RANKL, receptor activator of NF-κB ligand; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole.

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doi:10.1016/j.bcp.2003.11.011

in cancer prevention [6]. The effects of carotenoids on bone metabolism, however, have not been fully clarified.

Retinal (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 [7–9]. β-Cryptoxanthin is carotenoid which is greatly present in orange, and it is enzymatically converted from β-carotene (provitamin A) in plants. Of the various carotinoids (including β-cryptoxanthin, lutein, and lycopene) and rutin (quercetin-3-rutinoside), β-cryptoxanthin has been found to have a unique anabolic effect on bone calcification [10]. Moreover, β-cryptoxanthin has been shown to have a stimulatory effect on bone formation and an inhibitory effect on bone resorption in rat femoral tissue culture *in vitro* [11], suggesting that the carotenoid has a regulatory role in bone metabolism. The cellular mechanism by which β-cryptoxanthin has an anabolic effect on bone metabolism, however, remains to be elucidated.

The present study was undertaken to determine the action of β-cryptoxanthin on osteoclastic cell formation, which is related to stimulation of bone resorption, in bone marrow culture *in vitro*. Osteoclast-like cell formation is induced by bone-resorbing factors in bone marrow cultures [12]. We found that β-cryptoxanthin inhibits osteoclast-like cell formation induced by bone-resorbing factors in mouse marrow cultures *in vitro*.

2. Materials and methods

2.1. Chemicals

α-Minimal essential medium (α-MEM) and penicillin–streptomycin (5000 U/mL penicillin; 5000 μg/mL streptomycin) were obtained from Gibco Laboratories. Fetal bovine serum was obtained from Bioproducts Inc. Genistein, 1,25-dihydroxyvitamin D_3 (VD_3), prostaglandin E_2 (PGE_2), lipopolysaccharide (LPS), TNFα, 17β-estradiol, PMA, DcAMP, M-CSF (mouse), RANKL (mouse), cycloheximide, and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) were obtained from Sigma Chemical Co. Synthetic human PTH (1–34) and synthetic [$Asu^{1,7}$]eel calcitonin were supplied by Asahi Chemical Industry Co, Ltd. β-Cryptoxanthin was obtained from Extransynthese. Zinc sulfate and other chemicals were of reagent grade and were obtained from Wako Pure Chemical Industries. All water used was glass distilled.

2.2. Animals

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

2.3. Marrow culture

Bone marrow cells were isolated from mice, as reported elsewhere [12,13]. 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 at 1.0×10^7 cells/mL in 24-well plates (0.5 mL/well) in a water-saturated atmosphere containing 5% CO_2 and 95% air at 37°. The cells were cultured for 3 days; then 0.2 mL of the old medium was replaced with fresh medium, and the cultures were maintained for an additional 4 days. Various concentrations of β-cryptoxanthin were added to the culture medium containing either vehicle, PTH (10^{-7} M), PGE_2 (10^{-5} M), VD_3 (10^{-7} M), LPS (10 μg/mL), TNFα (10 ng/mL), or RANKL (10 or 20 ng/mL) plus M-CSF (10 or 20 ng/mL) with an effective concentration at the beginning of the cultures and at the time of medium change. In separate experiments, the respective media contained either calcitonin, 17β-estradiol, genistein, zinc sulfate, PMA, or DcAMP.

2.4. Enzyme histochemistry

After being cultured for 7 days, cells adherent to the 24-well plates were stained for tartrate-resistant acid phosphatase (TRACP), a marker enzyme of osteoclasts [14,15]. 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 [14]. The fixed cells were incubated for 12 min at room temperature (25°) 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 [14]. TRACP-positive multinucleated cells (MNCs) containing three or more nuclei were counted as osteoclast-like cells.

2.5. Pit formation assay

The pit formation assay was performed according to the method of Takada *et al.* [16] with some modifications. Briefly, transverse slices of dentine (150 μm in thickness) were prepared using a low-speed diamond saw (Leitz). Each slice was ground to 100 μm in thickness and sterilized in to ultrasonication to remove attached cells and subsequently stained with toluidine blue (0.1%, w/v). The number of pits formed on the slices was determined using a light microscope.

2.6. Statistical methods

Data were expressed as the mean ± SEM. Statistical differences were analyzed using Student's paired *t* test We

also used a multiple ANOVA to compare the treatment groups. A P value of less than 0.05 was considered to indicate a statistically significant difference.

3. Results

3.1. Effect of β-cryptoxanthin on bone-resorbing factor-induced osteoclast-like cell formation in marrow cell culture

The effect of β-cryptoxanthin on the bone-resorbing factor [17–21] induced osteoclast-like MNC formation in the mouse marrow culture system was examined. Mouse marrow cells were cultured for 7 days in medium containing either vehicle, PTH (10^{-7} M), PGE_2 (10^{-5} M), VD_3 (10^{-7} M), LPS (10 μg/mL medium), or TNFα (10 ng/mL) in the absence or presence of β-cryptoxanthin (10^{-9} to 10^{-6} M). The number of TRACP-positive MNCs was significantly increased in the presence of PTH (Fig. 1A), PGE_2 (Fig. 2A), VD_3 (Fig. 3A), LPS (Fig. 4A), or TNFα (Fig. 5A). TRACP-positive MNCs were not formed appreciably in the control culture without bone-resorbing factors at any incubation time. The presence of β-cryptoxanthin (10^{-8} to 10^{-6} M) in the culture medium caused a significant decrease in the number of TRACP-positive MNCs stimulated by PTH (Fig. 1B), PGE_2 (Fig. 2B), VD_3 (Fig. 3B), LPS (Fig. 4B), or TNFα (Fig. 5B). β-Cryptoxanthin in the range of 10^{-9} to 10^{-6} M did not have an inhibitory effect on the proliferation of marrow cells; this was independent of the presence of bone-resorbing factors (data not shown).

The effect of β-cryptoxanthin and other agents on PTH- or PGE_2-induced osteoclast-like MNC formation in mouse marrow culture were compared (Table 1). Mouse marrow cells were cultured for 7 days in medium containing either vehicle, calcitonin, 17β-estradiol, genistein, zinc sulfate, or β-cryptoxanthin at the indicated concentration. Each caused a significant inhibition of osteoclast-like MNC formation induced by PTH (10^{-7} M) or PGE_2 (10^{-7} M).

In another experiments, mouse marrow cells were cultured for 3 days in medium containing either vehicle, PTH (10^{-7} M), or PGE_2 (10^{-5} M); then β-cryptoxanthin (10^{-7} and 10^{-6} M) was added to the culture medium containing each bone-resorbing agent, and the cells were further

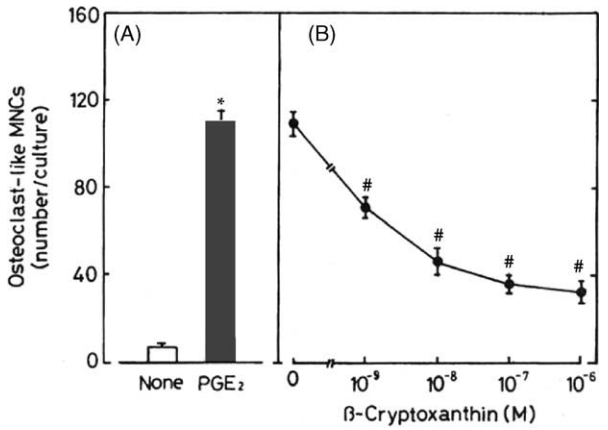

Fig. 2. Effect of β-cryptoxanthin on PGE_2-induced osteoclast-like cell formation in mouse marrow culture. Mouse marrow cells were cultured for 7 days in medium containing either vehicle, PGE_2 (10^{-5} M) or PGE_2 (10^{-5} M) plus β-cryptoxanthin (10^{-9} to 10^{-6} M). Cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. Each value is the mean ± SEM of five cultures. $^{*}P < 0.01$, compared with the control (none) value. $^{\#}P < 0.01$, compared with the value for PGE_2 alone. (Panel A) White bar, none or black bar, PGE_2. (Panel B) PGE_2 plus β-cryptoxanthin.

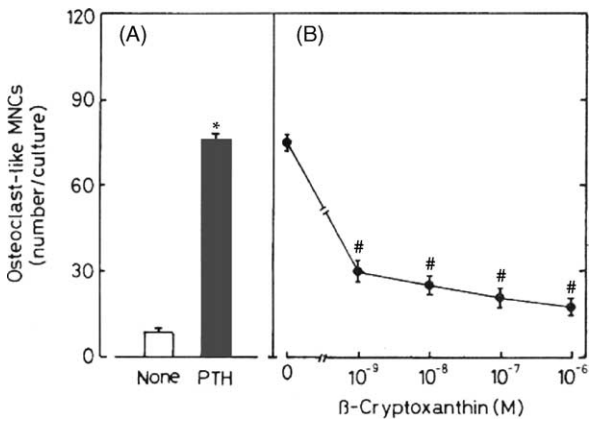

Fig. 1. Effect of β-cryptoxanthin on PTH-induced osteoclast-like cell formation in mouse marrow culture. Mouse marrow cells were cultured for 7 days in medium containing either vehicle, PTH (10^{-7} M) or PTH (10^{-7} M) plus β-cryptoxanthin (10^{-9} to 10^{-6} M). Cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. Each value is the mean ± SEM of five cultures. $^{*}P < 0.01$, compared with the control (none) value. $^{\#}P < 0.01$, compared with the value for PTH alone. (Panel A) White bar, none or black bar, PTH. (Panel B) PTH plus β-cryptoxanthin.

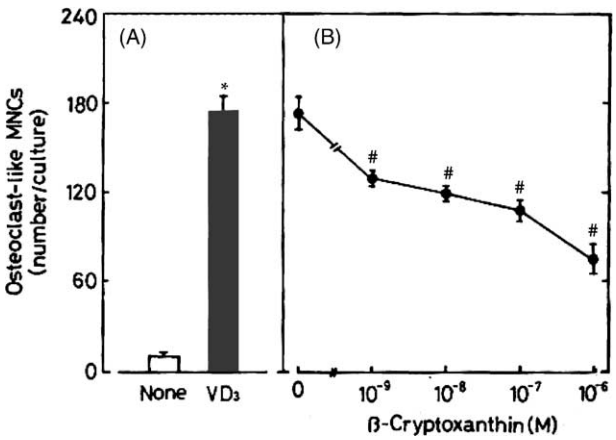

Fig. 3. Effect of β-cryptoxanthin on VD_3-induced osteoclast-like cell formation in mouse marrow culture. Mouse marrow cells were cultured for 7 days in medium containing either vehicle, VD_3 (10^{-7} M) or VD_3 (10^{-7} M) plus β-cryptoxanthin (10^{-9} to 10^{-6} M). Cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. Each value is the mean ± SEM of five cultures. $^{*}P < 0.01$, compared with the control (none) value. $^{\#}P < 0.01$, compared with the value for VD_3 alone. (Panel A) White bar, none or black bar, VD_3. (Panel B) VD_3 plus β-cryptoxanthin.

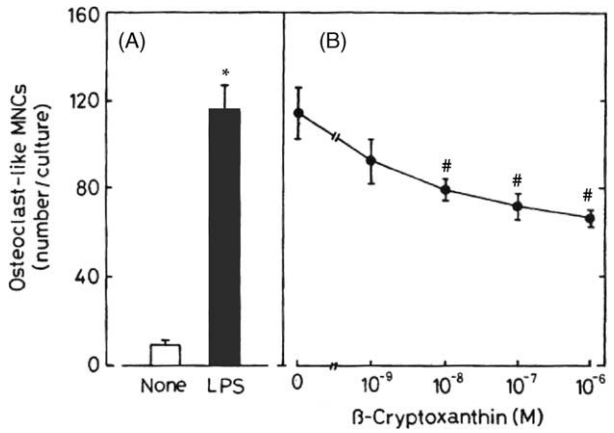

Fig. 4. Effect of β-cryptoxanthin on LPS-induced osteoclast-like cell formation in mouse marrow culture. Mouse marrow cells were cultured for 7 days in medium containing either vehicle, LPS (10 μg/mL) or LPS (10 μg/mL) plus β-cryptoxanthin (10^{-9} to 10^{-6} M). Cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. Each value is the mean ± SEM of five cultures. $^{*}P < 0.01$, compared with the control (none) value. $^{\#}P < 0.01$, compared with the value for LPS alone. (Panel A) White bar, none or black bar, LPS. (Panel B) LPS plus β-cryptoxanthin.

incubated for 4 days. In this case, the presence of β-cryptoxanthin caused a significant inhibition of osteoclast-like MNC formation induced by bone-resorbing agents (Fig. 6).

We also cultured unfractionated bone marrow cells on a dentine slice and examined the effect of β-cryptoxanthinon the number of total pit area as well as total pit number of bone resorption formed over days. β-Cryptoxanthin (10^{-6} M) significantly inhibited the PTH (10^{-7} M)- or PGE_2 (10^{-5} M)-induced increase in the number of area and number of pits formed on a dentine slice (data not shown).

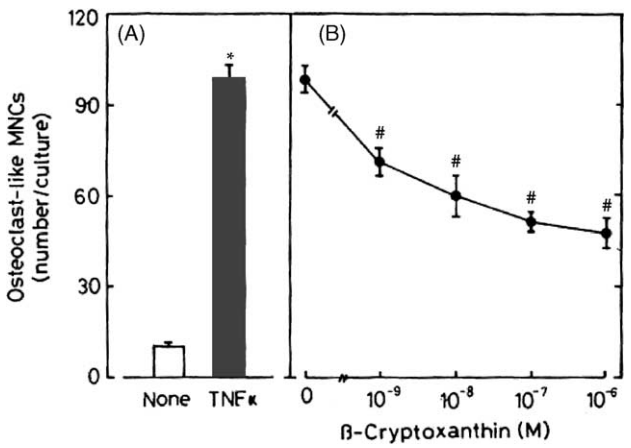

Fig. 5. Effect of β-cryptoxanthin on TNFα-induced osteoclast-like cell formation in mouse marrow culture. Mouse marrow cells were cultured for 7 days in medium containing either vehicle, TNFα (10 ng/mL) or TNFα (10 ng/mL) plus β-cryptoxanthin (10^{-9} to 10^{-6} M). Cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. Each value is the mean ± SEM of five cultures. $^{*}P < 0.01$, compared with the control (none) value. $^{\#}P < 0.01$, compared with the value for TNFα alone. (Panel A) White bar, none or black bar, TNFα. (Panel B) TNFα plus β-cryptoxanthin.

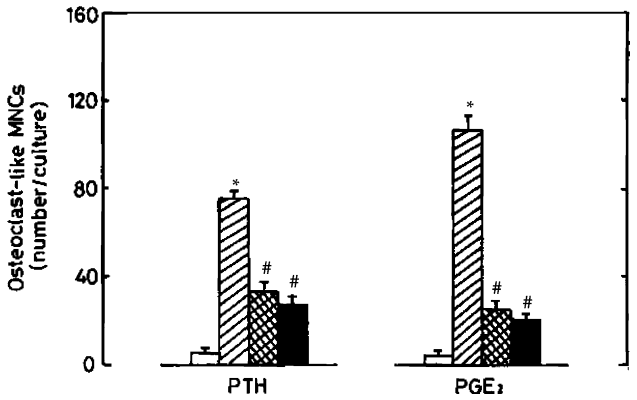

Fig. 6. Effect of β-cryptoxanthin at the later stage of osteoclast-like cell formation induced by PTH or PGE_2 in mouse marrow culture. Mouse marrow cells were cultured for 7 days in medium containing either vehicle, PTH (10^{-7} M) or PGE_2 (10^{-5} M). After 3 days of culture, the medium was changed, then β-cryptoxanthin (10^{-7} or 10^{-6} M) was added in the culture medium containing PTH (10^{-7} M) or PGE_2 (10^{-5} M), and the cells were cultured for an additional 4 days. Cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. Each value is the mean ± SEM of five cultures. $^{*}P < 0.01$, compared with the control (none) value. $^{\#}P < 0.01$, compared with the value for PTH or PGE_2 alone. White bars, control (none); hatched bars, PTH or PGE_2 alone; double hatched, PTH or PGE_2 plus β-cryptoxanthin (10^{-7} M); black bars, PTH or PGE_2 plus β-cryptoxanthin (10^{-6} M).

3.2. Effect of β-cryptoxanthin on intracellular signaling factor-induced osteoclast-like cell formation in marrow cell culture

The effect of DcAMP or PMA on osteoclast-like MNC formation in mouse culture was examined. The effect of

Table 1
Comparison of the effects of β-cryptoxanthin and other agents on the PTH- or PGE_2-induced osteoclast-like cell formation in mouse marrow culture

Treatment	Osteoclast-like MNCs (number/culture)	
	PTH	PGE_2
Control	71.8 ± 2.6	110.0 ± 5.2
Calcitonin		
10^{-10} M	34.6 ± 6.0*	89.7 ± 2.2*
10^{-9} M	32.8 ± 6.1*	70.0 ± 2.0*
17β-Estradiol		
10^{-10} M	48.0 ± 3.8*	88.0 ± 2.0*
10^{-9} M	37.7 ± 4.3*	81.5 ± 4.8*
Genistein		
10^{-7} M	44.3 ± 4.6*	75.5 ± 3.0*
10^{-6} M	46.1 ± 6.7*	69.8 ± 3.7*
Zinc sulfate		
10^{-5} M	37.6 ± 5.6*	71.5 ± 3.7*
10^{-4} M	26.1 ± 3.0*	67.0 ± 4.7*
β-Cryptoxanthin		
10^{-7} M	25.7 ± 2.2*	28.8 ± 2.3*
10^{-6} M	20.1 ± 2.0*	25.0 ± 3.7*

Mouse marrow cells were cultured for 7 days in medium containing either vehicle, PTH (10^{-7} M) or PGE_2 (10^{-7} M) in the absence or presence of various inhibitors. Each value is the mean ± SEM of five cultures.
* $P < 0.01$, compared with the control value of PTH or PGE_2 alone.

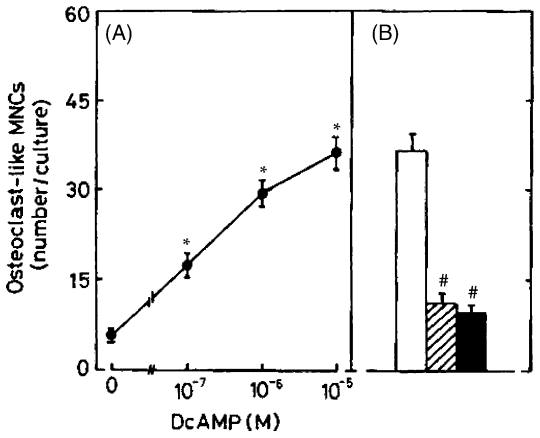

Fig. 7. Effect of β-cryptoxanthin on DcAMP-induced osteoclast-like cell formation in mouse marrow culture. (Panel A) Mouse marrow cells were cultured for 7 days in medium containing either vehicle or DcAMP (10^{-7} to 10^{-5} M). (Panel B) After 3 days of culture, the medium was changed, then β-cryptoxanthin (10^{-7} or 10^{-6} M) was added in the culture medium containing DcAMP (10^{-5} M), and the cells were cultured for an additional 4 days. Cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. Each value is the mean ± SEM of five cultures. $^{*}P < 0.01$, compared with the control (none) value. $^{\#}P < 0.01$, compared with the value for DcAMP (10^{-5} M). White bars, DcAMP (10^{-5} M); hatched bars, DcAMP (10^{-5} M) plus β-cryptoxanthin (10^{-7} M); black bars, DcAMP (10^{-5} M) plus β-cryptoxanthin (10^{-6} M).

PTH or PGE_2 is mediated through cyclic AMP [22,23]. PMA is an activator of protein kinase C, which is related to intracellular Ca^{2+} signaling [24]. Mouse marrow cells were cultured for 7 days in medium containing either vehicle, DcAMP (10^{-7} to 10^{-5} M) or PMA (10^{-7} to 10^{-5} M) in the absence or presence of β-cryptoxanthin

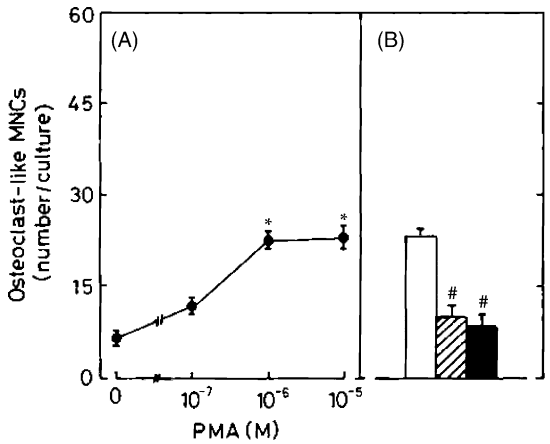

Fig. 8. Effect of β-cryptoxanthin on PMA-induced osteoclast-like cell formation in mouse marrow culture. (Panel A) Mouse marrow cells were cultured for 7 days in medium containing either vehicle or PMA (10^{-7} to 10^{-5} M). (Panel B) After 3 days of culture, the medium was changed, then β-cryptoxanthin (10^{-7} or 10^{-6} M) was added in the culture medium containing PMA (10^{-5} M), and the cells were cultured for an additional 4 days. Cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. Each value is the mean ± SEM of five cultures. $^{*}P < 0.01$, compared with the control (none) value. $^{\#}P < 0.01$, compared with the value for PMA (10^{-5} M). White bars, PMA (10^{-5} M); hatched bars, PMA (10^{-5} M) plus β-cryptoxanthin (10^{-7} M); black bars, PMA (10^{-5} M) plus β-cryptoxanthin (10^{-6} M).

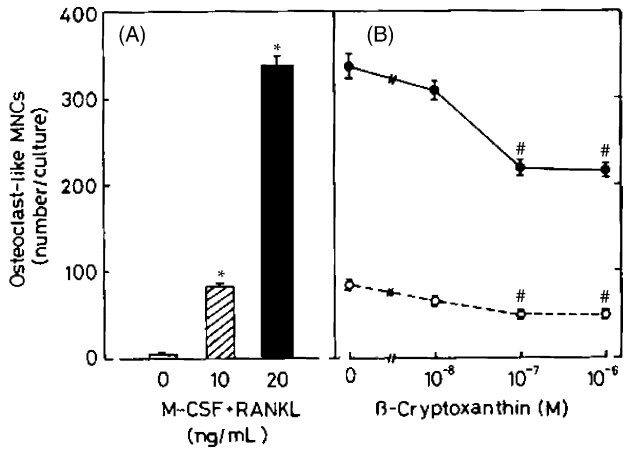

Fig. 9. Effect of β-cryptoxanthin on RANKL-induced osteoclast-like cell formation in mouse marrow culture. (Panel A) Mouse marrow cells were cultured for 7 days in medium containing either vehicle, RANKL (10 ng/mL) plus M-CSF (10 ng/mL) or RANKL (20 ng/mL) plus M-CSF (20 ng/mL). (Panel B) After 3 days of culture, the medium changed, then β-cryptoxanthin (10^{-8} or 10^{-6} M) was added in the culture medium containing RANKL plus M-CSF (10 or 20 ng/mL), and the cells were cultured for an additional 4 days. Cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. Each value is the mean ± SEM of five cultures. $^{*}P < 0.01$, compared with the control (none) value. $^{\#}P < 0.01$, compared with the value for RANKL plus M-CSF. (Panel A) White bar, none; hatched bar, RANKL (10 ng/mL) plus M-CSF (10 ng/mL), black bar, RANKL (10 ng/mL) plus M-CSF (10 ng/mL). (Panel B) Open circles, RANKL (10 ng/mL) plus M-CSF (10 ng/mL); closed circles, RANKL (20 ng/mL) plus M-CSF (20 ng/mL).

(10^{-7} and 10^{-6} M). Osteoclast-like cell formation was significantly elevated in the presence of DcAMP (10^{-7} to 10^{-5} M) (Fig. 7A) or PMA (10^{-6} and 10^{-5} M) (Fig. 8A) The effect of DcAMP or PMA at 10^{-5} M was remarkable. This effect was significantly inhibited by the addition of β-cryptoxanthin (10^{-7} and 10^{-6} M) (Figs. 7B or 8B).

3.3. Effect of β-cryptoxanthin on RANKL-induced osteoclast-like cell formation in marrow cell culture

The effect of β-cryptoxanthin on RANKL and M-CSF-induced osteoclast-like MNC formation in mouse marrow culture was examined. RANKL and M-CSF are critical cytokines that stimulate osteoclast differentiation [25,26]. Mouse marrow cells were cultured for 7 days in medium containing either vehicle or RANKL plus M-CSF at 10 or 20 ng/mL medium. The number of TRACP-positive MNCs was markedly increased in the presence of RANKL plus M-CSF (Fig. 9A). Mouse marrow cells were cultured for 3 days in medium containing either vehicle or RANKL plus M-CSF at 10 or 20 ng/mL; then β-cryptoxanthin (10^{-8} to 10^{-6} M) was added to the culture medium containing the cytokines, and the cells were further incubated for 4 days. In this case, the presence of β-cryptoxanthin (10^{-7} to 10^{-6} M) caused a significant inhibition of osteoclast-like MNC formation induced by the cytokines (Fig. 9B).

The effect of PMA and DcAMP on RANKL and M-CSF-induced osteoclast-like MNC formation in mouse

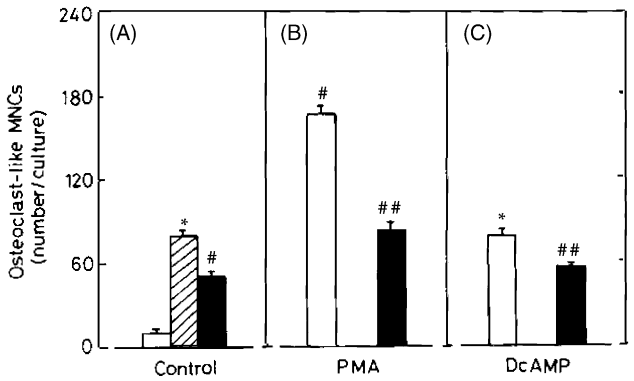

Fig. 10. Effect of β-cryptoxanthin on RANKL-induced osteoclast-like cell formation with or without PMA or DcAMP addition in mouse marrow culture. Mouse marrow cells were cultured for 7 days in medium containing either vehicle or RANKL (10 ng/mL) plus M-CSF (10 ng/mL). After 3 days of culture, the medium was changed, then β-cryptoxanthin (10^{-6} M), PMA (10^{-5} M), PMA (10^{-5} M) plus β-cryptoxanthin (10^{-6} M), DcAMP (10^{-5} M), or DcAMP (10^{-5} M) plus β-cryptoxanthin (10^{-6} M) was added in the culture medium containing RANKL (10 ng/mL) plus M-CSF (10 ng/mL) and the cells were cultured for an additional 4 days. Cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. Each value is the mean ± SEM of five cultures. $^{*}P < 0.01$, compared with the control (none) value. $^{\#}P < 0.01$, compared with the value for RANKL plus M-CSF. $^{\#\#}P < 0.01$, compared with the value for RANKL plus M-CSF with PMA or RANKL plus M-CSF with DcAMP. (Panel A) White bar, none; hatched bar, RANKL plus M-CSF; black bar, RANKL plus M-CSF with β-cryptoxanthin. (Panel B) White bar, RANKL plus M-CSF with PMA; black bar, RANKL plus M-CSF with PMA and β-cryptoxanthin. (Panel C) White bar, RANKL plus M-CSF with DcAMP; black bar, RANKL plus M-CSF with DcAMP and β-cryptoxanthin.

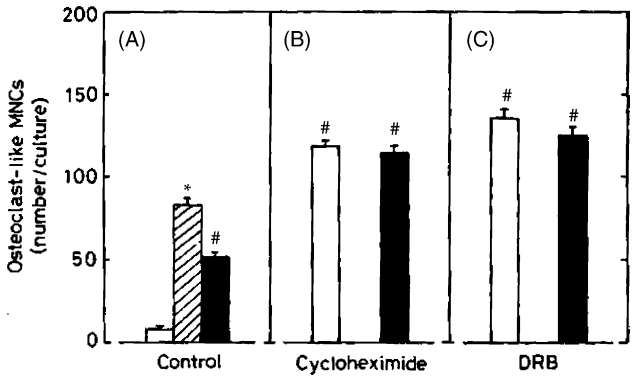

Fig. 11. Effect of β-cryptoxanthin on RANKL-induced osteoclast-like cell formation with or without cycloheximide or DRB addition in mouse marrow cultures. Mouse marrow cells were cultured for 7 days in medium containing either vehicle or RANKL (10 ng/mL) plus M-CSF (10 ng/mL). After 3 days of culture, the medium was changed, then β-cryptoxanthin (10^{-6} M), cycloheximide (10^{-7} M), cycloheximide (10^{-7} M) plus β-cryptoxanthin (10^{-6} M), DRB (10^{-6} M), or DRB (10^{-6} M) plus β-cryptoxanthin (10^{-6} M) was added in the culture medium containing RANKL (10 ng/mL) plus M-CSF (10 ng/mL), and the cells were cultured for an additional 4 days. Cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. Each value is the mean ± SEM of five cultures. $^{*}P < 0.01$, compared with the control (none) value. $^{\#}P < 0.01$, compared with the value for RANKL plus M-CSF. (Panel A) White bar, none; hatched bar, RANKL plus M-CSF; black bar, RANKL plus M-CSF with β-cryptoxanthin. (Panel B) White bar, RANKL plus M-CSF with cycloheximide; black bar, RANKL plus M-CSF with cycloheximide and β-cryptoxanthin. (Panel C) White bar RANKL plus M-CSF with DRB; black bar, RANKL plus M-CSF with DRB and β-cryptoxanthin.

marrow culture was examined. The effect of RANKL (10 ng/mL) and M-CSF (10 ng/mL) in stimulating TRACP-positive MNC formation was markedly enhanced in the presence of PMA (10^{-5} M) (Fig. 10B). Such an effect was not seen in the presence of DcAMP (10^{-5} M) (Fig. 10C). The addition of β-cryptoxanthin (10^{-6} M) caused a significant inhibition of TRACP-positive MNC formation enhanced by PMA in the presence of RANKL plus M-CSF (Fig. 10B).

The effect of β-cryptoxanthin on RANKL plus M-CSF-induced osteoclast-like MNC formation in mouse marrow culture was examined in the presence of cycloheximide, an inhibitor of translational process, and DRB, an inhibitor of transcription. Interestingly, the presence of cycloheximide (10^{-7} M) or DRB (10^{-6} M) caused a significant increase in TRACP-positive MNC formation in the presence of RANKL (10 ng/mL) and M-CSF (10 ng/mL) (Fig. 11B and C). The effect of β-cryptoxanthin in inhibiting RANKL plus M-CSF-induced TRACP-positive MNC formation was not seen in the presence of cycloheximide or DRB (Fig. 11B and C).

Meanwhile, the effect of PTH (10^{-7} M) (Fig. 12) or TNFα (10 ng/mL) (Fig. 13) in stimulating TRACP-positive MNC formation in mouse marrow culture was not significantly altered in the presence of cycloheximide (10^{-7} M) or DRB (10^{-6} M). The inhibitory effect of

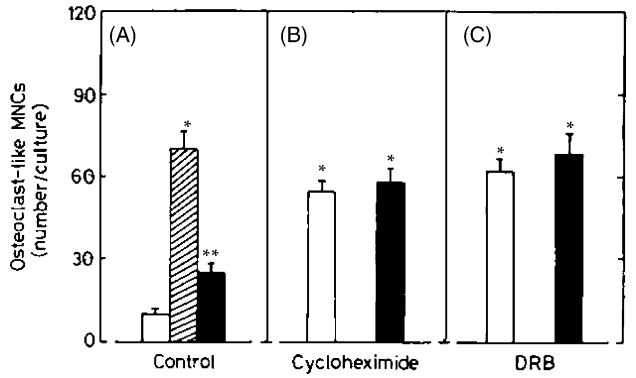

Fig. 12. Effect of β-cryptoxanthin on PTH-induced osteoclast-like cell formation with or without cycloheximide or DRB addition in mouse marrow culture. Mouse marrow cells were cultured for 7 days in medium containing either vehicle or PTH (10^{-7} M). After 3 days of culture, the medium was changed, then β-cryptoxanthin (10^{-6} M), cycloheximide (10^{-7} M), cycloheximide (10^{-7} M) plus β-cryptoxanthin (10^{-6} M), DRB (10^{-6} M), or DRB (10^{-6} M) plus β-cryptoxanthin (10^{-6} M) was added in the culture medium containing PTH (10^{-7} M), and the cells were cultured for an additional 4 days. Cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. Each value is the mean ± SEM of five cultures. $^{*}P < 0.01$, compared with the control (none) value. $^{**}P < 0.01$, compared with the value for PTH alone. (Panel A) White bar, none; hatched bar, PTH; black bar, PTH plus β-cryptoxanthin. (Panel B) White bar, PTH plus cycloheximide; black bar, PTH plus cycloheximide with β-cryptoxanthin. (Panel C) White bar, PTH plus DRB; black bar, PTH plus DRB with β-cryptoxanthin.

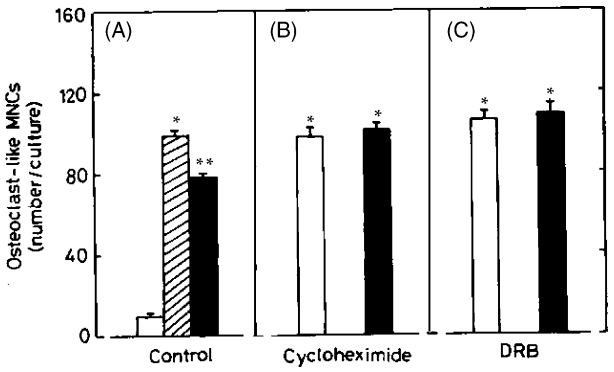

Fig. 13. Effect of β-cryptoxanthin on TNFα-induced osteoclast-like cell formation with or without cycloheximide or DRB addition in mouse marrow cultures. Mouse marrow cells were cultured for 7 days in medium containing either vehicle or TNFα (10 ng/mL). After 3 days of culture, the medium was changed, then β-cryptoxanthin (10^{-6} M) cycloheximide (10^{-7} M), cycloheximide (10^{-7} M) plus β-cryptoxanthin (10^{-6} M), DRB (10^{-6} M), or DRB (10^{-6} M) plus β-cryptoxanthin (10^{-6} M) was added in the culture medium containing TNFα (10 ng/mL), and the cells were cultured for an additional 4 days. Cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. Each value is the mean ± SEM of five cultures. $^{*}P < 0.01$, compared with the control (none) value. $^{**}P < 0.01$, compared with the value for TNFα alone. (Panel A) White bar, none; hatched bar, TNFα; black bar, TNFα plus cryptoxanthin. (Panel B) White bar, TNFα plus cycloheximide; black bar, TNFα plus cycloheximide with β-cryptoxanthin. (Panel C) White bar, TNFα plus DRB; black bar, TNFα plus DRB with β-cryptoxanthin.

β-cryptoxanthin- or DRB (10^{-7} M)- or TNFα (10 ng/mL)-induced TRACP-positive MNC formation was not seen in the presence of cycloheximide (10^{-7} M) or DRB (10^{-6} M) (Figs. 12 and 13).

4. Discussion

This study demonstrates that β-cryptoxanthin has an inhibitory effect on osteoclast-like cell formation in mouse marrow cultures *in vitro*. The inhibitory effect of β-cryptoxanthin was found to be equal to that of 17β-estradiol, calcitonin, genistein, and zinc sulfate, which can inhibit osteoclast-like cell formation induced by bone-resorbing factors [21,27]. β-Cryptoxanthin has been shown to have an inhibitory effect on bone resorption in rat femoral tissues *in vitro* [11]. The present finding supports the view that β-cryptoxanthin can inhibit osteoclastic bone resorption *in vitro*.

The inhibitory effect of β-cryptoxanthin on osteoclast-like cell formation was greatly seen at the later stage of osteoclast differentiation in bone marrow cultures, suggesting that the carotenoid has a potent effect on the process of differentiation from mononuclear osteoclast to osteoclast. RANKL acts on osteoclast progenitors, and the cytokine stimulates osteoclast differentiation [25,26]. RANKL plays a pivotal role in osteoclast differentiation. RANKL expression is induced in osteoblastic cells and bone marrow stromal cells in response to osteoporotic factors, such as PTH, PGE_2, and VD_3, and combined treatment of hematopoietic cells with M-CSF and the soluble form of RANKL (sRANKL) induced osteoclast differentiation *in vitro* [28]. The receptor protein RANK is expressed on the surface of osteoclast progenitors [29]. A soluble fragment containing part of the extracellular domain of RANKL (the carboxyterminal half of the protein, amino acids 158–316), is capable of promoting osteoclastogenesis in the presence of M-CSF. The supporting cells (osteoblasts or stromal cells) are not required for this activity [30]. β-Cryptoxanthin significantly inhibited osteoclast-like cell formation induced by PTH, PGE_2, and VD_3 in mouse marrow cultures. Presumably, the inhibitory effect of the carotenoid is partly involved in RANKL expression which is related to the effect of PTH, PGE_2, or VD_3.

LPS- or TNFα-induced osteoclast-like cell formation in mouse marrow cultures was significantly prevented by β-cryptoxanthin. TNFα is an autocrine factor in osteoclasts, promoting their differentiation, and mediates RANKL's induction of osteoclastogenesis [31]. TNFα has also been shown to mediate *via* its p55 receptor in the LPS-stimulated osteoclastogenesis [32]. The inhibitory effect of β-cryptoxanthin on the TNFα or LPS-stimulated osteoclastogenesis are probably a combination of effects by locally produced RANKL and LPS or TNFα.

The interaction of RANKL with its receptor RANK leads to the recruitment of the signaling adaptor molecules TRAFs (TNF receptor-associated factors) to the receptor complex and the activation of nuclear factor-kappa B (NF-κB) and c-Jun N-terminal kinase (JNK) [33–36]. Protein kinase C family enzyme has a role in regulation of osteoclast formation and function potentially by participating in the extracellular signal-regulated kinase (ERK) signaling pathway of M-CSF and RANKL [37]. PMA is an activator of protein kinase C [24]. PMA significantly stimulated osteoclast-like cell formation in mouse marrow cultures, and the PMA-induced osteoclastogenesis was significantly inhibited by β-cryptoxanthin. Moreover, β-cryptoxanthin was found to have a significant inhibitory effect on DcAMP-induced osteoclast-like cell formation in mouse marrow cultures. From these results, it is assumed that activation of protein kinase C and protein kinase A pathway leads to increased RANKL expression, and that β-cryptoxanthin can inhibit protein kinase C- or protein kinase A-related RANKL expression in osteoclastogenesis.

Osteoclast-like cell formation in mouse marrow cultures was markedly stimulated in the presence of RANKL and M-CSF, indicating that RANKL induced osteoclastogenesis. β-Cryptoxanthin was found to inhibit osteoclastogenesis induced by RANKL and M-CSF The presence of PMA markedly enhanced RANKL and M-CSF-induced osteoclastogenesis in mouse marrow cultures. The finding suggests that RANKL stimulation in osteoclastogenesis is modulated by activation of protein kinase C. β-Cryptoxanthin may have an inhibitory effect on osteoclastogenesis which is related to RANKL expression in the presence of M-CSF.

The effect of β-cryptoxanthin in inhibiting PTH-, TNFα-, or RANKL-induced osteoclast-like cell formation in mouse marrow culture was completely blocked in the presence of cycloheximide, an inhibitor of protein synthesis at translational process, or DRB, an inhibitor of transcriptional process. The stimulatory effect of PTH, TNFα, or RANKL on osteoclastogenesis was not inhibited in the presence of cycloheximide or DRB. Interestingly, the effect of RANKL was significantly enhanced in the presence of cycloheximide or DRB. It is speculated that RANKL stimulation on osteoclastogenesis induces a suppressor protein for osteoclastogenesis, and that regulates osteoclast differentiation by an autocrine mechanism. Presumably, the inhibitory effect of β-cryptoxanthin on osteoclastogenesis is partly involved in a newly synthesized protein component which induces a suppressor protein. Whether β-cryptoxanthin has an effect on expression of osteoprotegerin (OPG), a regulated suppressor of osteoclast differentiation [25,26], is unknown, however.

The effect of β-cryptoxanthin on osteoclast apoptosis has not been clarified. If β-cryptoxanthin has a stimulatory effect on osteoclast apoptosis, the carotenoid may induce a decrease in number of osteoclasts. This remains to be elucidated.

β-Cryptoxanthin in the range of 10^{-8} to 10^{-6} M caused a significant increase in calcium content in rat femoral tissues *in vitro* [10], and the carotenoid (10^{-8} to 10^{-6} M) had a significant inhibitory effect on various bone-resorbing factors-induced bone resorption in rat femoral tissues *in vitro* [11]. Moreover, β-cryptoxanthin (10^{-8} to 10^{-6} M) was found to have a significant inhibitory effect on osteoclast-like cell formation in mouse marrow cultures. β-Cryptoxanthin is abundant in orange juice. It has been reported that the serum concentrations of β-cryptoxanthin due to consumption of vegetable juice in woman is present in the range 1.3×10^{-7} to 5.3×10^{-7} M [38]. The intake of β-cryptoxanthin may have a role in the prevention of bone loss with increasing age.

In conclusion, it has been demonstrated that the carotenoid β-cryptoxanthin has an inhibitory effect on osteoclast-like cell formation induced by various factors in stimulating osteoclastogenesis in mouse marrow cultures *in vitro*. β-Cryptoxanthin may act as an inhibitor in RANKL stimulation which induces osteoclastogenesis.

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