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ARTICLE

Fucoxanthin Induces Apoptosis in Osteoclast-like Cells Differentiated from RAW264.7 Cells

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Fucoxanthin is an oxygenated carotenoid present in edible brown sea algae, and dietary fucoxanthin is recognized to exhibit various beneficial effects. In the present study, the effects of fucoxanthin on osteoclastogenesis were investigated using cells from the macrophage cell line RAW264.7, which have the capacity to differentiate into osteoclast-like cells when stimulated by receptor activator of nuclear factor κB ligand. Fucoxanthin significantly suppressed the differentiation of RAW264.7 cells at 2.5 μM, which was not toxic to RAW264.7 cells. Treatment with 2.5 μM fucoxanthin also induced apoptosis accompanied by activation of caspase-3 in osteoclast-like cells. On the other hand, 2.5 μM fucoxanthin did not decrease cell viability in cells of the osteoblast-like cell line MC3T3-E1, indicating that the apoptosis-inducing activity of fucoxanthin in osteoclasts is stronger than that in osteoblasts. The present in vitro study suggests that fucoxanthin suppresses osteoclastogenesis via the inhibition of osteoclast differentiation and the induction of apoptosis in osteoclasts, but not bone formation.

KEYWORDS: Fucoxanthin; apoptosis; osteoclasts; RAW264.7 cells; receptor activator of nuclear factor κB ligand (RANKL); osteoclastogenesis; *Laminaria japonica*

INTRODUCTION

Osteoclasts, large multinucleated cells, resorb bone, maintain calcium homeostasis, and support normal bone remodeling (*1*). However, excess osteoclast activity leads to an imbalance in bone remodeling, which favors resorption (*2*) and induces osteoporosis, periodontal disease, rheumatoid arthritis, multiple myeloma, and metastatic cancers (*3*). Osteoclasts are formed through the fusion of mononuclear precursors by a multistep differentiation process under the control of the bone microenvironment, which includes stromal cells, osteoblasts, and local factors (*3*). In vitro studies found that at least two extracellular factors, macrophage-colony stimulating factor and receptor activator of nuclear factor κB ligand (RANKL), are implicated in the differentiation of osteoblasts to osteoclasts (*4*−*8*). Other factors, such as interleukin (IL)-1, IL-6, tumor necrosis factor γ, and transforming growth factor β, also affect osteoclast differentiation. It is feasible to use in vitro models to modulate osteoclast differentiation and predict the effects on bone resorption in vivo, and RAW264.7 cells in the presence of RANKL differentiate readily into pure populations of osteoclast-like cells (*9*). These multinucleated cells express known osteoclast markers and exhibit the capacity to form resorption pits on mineralized surfaces. As precursor cells differentiate and fuse to form mature osteoclasts, signature phenotype markers are expressed, including tartrate-resistant acid phosphatase (TRAP) (*1*, *10*).

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Fucoxanthin is an oxygenated carotenoid produced specifically by edible brown sea algae such as kombu (*Laminaria japonica*), wakame (*Undaria pinnatifida*), and arame (*Eisenia bicyclis*) and has received considerable attention due to its scientifically proven beneficial effects on human health: suppressive effects on adipocyte differentiation (*11*); antimutagenicity (*12*); antiocular inflammation effects (*13*); and preventive effects on colon, liver, and lung cancers (*14*−*17*). However, no studies have been published about the effects of fucoxanthin on bone health, although a number of studies on this subject have investigated the effects of other carotenoids. Lycopene, which is abundant in tomatoes, exhibits inhibitory effects on osteoclast formation and resorption (*18*, *19*) as well as stimulatory effects on cell proliferation and alkaline phosphatase, a differentiation marker of osteoblasts (*20*, *21*). β-Cryptoxanthin, which is abundant in citrus fruits, shows anabolic effects on bone calcification (*22*), stimulates bone formation, and inhibits bone resorption in cultured bone tissues in vitro (*23*). In rats with streptozotocin-induced diabetes and in ovariectomized rats, β-cryptoxanthin also prevented bone loss (*24*) and had anabolic effects on bone components (*25*, *26*). We predict that fucoxanthin also possesses anti-osteoporotic effects.

In the present study, we examined the effects of fucoxanthin on the osteoclast differentiation of precursor RAW264.7 monocytes and its cytotoxicity against osteoclast-like cells differentiated from RAW264.7 cells and cells of the osteoblast-like cell line MC3T3-E1.

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MATERIALS AND METHODS

Chemicals. Propidium iodide (PI), ribonuclease A (RNase A), proteinase K, 4′,6-diamino-2-phenylindole (DAPI), and recombinant mouse soluble receptor activator of nuclear factor κB ligand (RANKL) were purchased from Sigma Chemicals (St. Louis, MO). For the cell culture, Dulbecco's modified Eagle's medium (DMEM) was purchased from Nissui Pharmaceutical (Tokyo, Japan), α-minimal essential medium (α-MEM) was from Wako Pure Chemical Industries (Osaka, Japan), and fetal bovine serum (FBS) was from Sigma Chemicals.

Preparation of Fucoxanthin. The fucoxanthin used in this study was isolated from the brown sea algae *L. japonica* (kombu) as described previously (*27*). The purity of the fucoxanthin used in this study was >95% as determined by HPLC. Fucoxanthin was dissolved in dimethyl sulfoxide (DMSO) at 10 mM as the stock solution.

Cell Culture. Cells of the murine macrophage cell line RAW264.7 and cells of the osteoblast-like cell line MC3T3-E1 were maintained in DMEM containing 10% heat-inactivated FBS, 4 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere of 95% air−5% CO_2 at 37 °C.

Determination of Cell Viability. Cell viability was determined using the trypan blue dye exclusion assay. RAW264.7 cells (2×10^3) were seeded onto 60 mm dishes, incubated for 12 h for adherence, and treated with fucoxanthin for 4 days. Then, the cells were harvested by trypsinization and stained with 0.4% (w/v) trypan blue (Gibco, Grand Island, NY) in phosphate-buffered saline (PBS). The trypan blue-unstained cells were counted using a hemocytometer under a phase-contrast microscope as viable cells. MC3T3-E1 cells (1×10^6) were seeded onto 100 mm dishes, and cell viability was determined according to the same method as used for the RAW264.7 cells.

Osteoclast Differentiation. RAW264.7 cells were seeded at a density of 6×10^3 cells/mL in α-MEM containing 10% FBS, that is, 6×10^2 cells in a 96-well plate, 6×10^3 cells in a 12-well plate, and 6×10^4 cells in a 100 mm dish. After the cells had adhered, the medium was replaced with fresh medium, and the cells were treated with 50 ng/mL of recombinant mouse soluble RANKL and cultured for 4 days to induce osteoclast differentiation. TRAP expression, a phenotype biomarker of osteoclasts, was determined using an acid phosphatase-staining kit (TRACP & ALP double-stain Kit, Takara Bio, Shiga, Japan) according to the manufacturer's protocol. The number of TRAP-positive multinucleated cells containing more than three nuclei was counted using a phase-contrast microscope as an index of mature osteoclast-like cells that had differentiated from RAW264.7 cells.

Apoptosis Analyses. In general, the apoptotic cells initially shrunk and then fragmented to form small apoptotic bodies (*28*). Apoptosis analyses were performed by morphological observation and the detection of DNA fragmentation in osteoclast-like cells differentiated from RAW264.7 cells.

For the morphological observation, the cells were washed with PBS and fixed with 70% ice-cold ethanol for at least 2 h at −20 °C. The fixed cells were then washed with PBS and incubated with 1 mg/mL RNase A and 50 μg/mL PI in PBS at room temperature for 30 min in the dark. The PI-stained nuclei were observed under a fluorescent microscope.

DNA fragmentation was measured as described previously (*29*). Briefly, the cells in the 12-well plate were lysed with 200 μL of TE buffer [10 mM Tris-HCl (pH 8.0) and 1 mM ethylenediaminetetraacetic acid (EDTA)] containing 0.5% Triton X-100, and the lysates were centrifuged at 17000*g* for 20 min at 4 °C to separate fragmented from intact DNA. The supernatant containing fragmented DNA was transferred to another microtube, and the pellet containing intact DNA was dissolved in 200 μL of TE buffer containing 0.5% SDS. Both fractions were treated with 0.25 mg/mL RNase A at 50 °C for 10 min and then with 0.25 mg/mL proteinase K at 50 °C for 30 min. DNA was precipitated with 42 mM NaCl and 50% isopropanol for 6 h at −20 °C, obtained by centrifugation at 17000*g* for 20 min, and labeled with 1 μg/mL of DAPI in Tris-NaCl buffer [10 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 5 mM EDTA] for 30 min. The fluorescent intensity was measured at 350 nm wavelength for excitation and at 452 nm for emission. DNA fragmentation was expressed as a percentage of fragmented DNA in the sum of intact and fragmented DNA.

For agarose gel electrophoresis, the fragmented DNA extracted by the precipitation with 50% isopropanol as described above was washed with ice-cold 70% ethanol and dissolved in 10 μL of TE buffer. The solution was applied to 2% agarose gel electrophoresis, and fragmented DNA was visualized by ethidium bromide staining with an UV transilluminator.

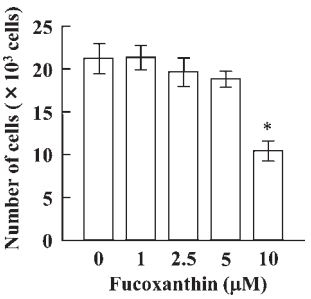

Figure 1. Effect of fucoxanthin on the viability of RAW264.7 cells. Cells were treated with the indicated concentrations of fucoxanthin for 4 days. The viable cells were evaluated by the trypan blue exclusion assay. The data are representative of three independent experiments and are shown as the mean ± SD of the three experiments (n = 6 culture dishes); *, $p < 0.05$ versus fucoxanthin-untreated cells (0 μM).

Western Blotting Analysis. Cells in the 100 mm dish were washed with PBS and lysed with RIPA buffer containing 50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholic acid, and 0.05% SDS. The protein concentration of the cell lysate was measured according to the Lowry method (*30*). Aliquots of 50 μg were subjected to SDS-PAGE and transferred onto poly(vinylidene difluoride) membranes (Amersham Biosciences, Piscataway, NJ). The sources of the primary antibodies used in this study were as follows: those for cleaved caspase-3 (Asp175), caspase-3, and PARP were from Cell Signaling Technology (Beverly, MA), and that for β-actin was from Santa Cruz Biotechnology (Santa Cruz, CA). As a secondary antibody, horseradish peroxidase conjugated anti-rabbit IgG and anti-mouse IgG were obtained from Amersham Biosciences and Nacali Tesque (Kyoto, Japan), respectively. The immunocomplexes were detected by enhanced chemiluminescence using the ECL Plus Western Blotting System (Amersham Biosciences).

Mesurement of Fucoxanthin and Fucoxanthinol in Osteoclast-like Cells. Amounts of fucoxanthinol and fuxanthinol in osteoclast-like cells were measured as described previously (*31*) with a slight modification. Briefly, osteoclast-like cells in 12-well plates were lysed with 1 mL of TE buffer containing 0.5% Triton X-100, and the lysates were centrifuged at 17000*g* for 10 min at 4 °C. The supernatant was mixed with the same volume of ethyl acetate. The ethyl acetate layer was collected and dried by nitrogen gas. The residue was dissolved in 200 μL of dichloromethane/methanol (1:4, v/v), and 20 μL of the solution was subjected to HPLC analysis. The HPLC system equipped with an L-7420 detector was employed. The column (250 mm ×4.6 mm internal diameter) and a guard column (10 × 4.0 mm internal diameter) were of the Capcell pak C18 UG80 (Shiseido, Tokyo, Japan) and were maintained at 35 °C. The solvent used was acetonitrile/methanol/water (75:15:10, v/v/v) containing 1 mg/mL ammonium acetate, and the flow rate was 1.0 mL/min. Fucoxanthin and fucoxanthinol were monitored at a wavelength of 450 nm and identified by retention time by comparison with standards of fucoxanthin and fucoxanthinol. These amounts were quantified from their peak areas by their standard curves.

Statistical Analysis. The data are reported as the mean ± standard deviation (SD). Statistical analysis was performed using Student's t test. Probability values of < 0.05 were considered to be statistically significant.

RESULTS

Effect of Fucoxanthin on the Viability of RAW264.7 Cells. The cytotoxicity of fucoxanthin on RAW264.7 cells was examined by the trypan blue exclusion assay (**Figure 1**). RAW264.7 cells were cultured for 4 days in media containing various concentrations of fucoxanthin. At concentrations of < 5 μM, fucoxanthin did not have detrimental effects on the survival of cells. However, 10 μM fucoxanthin caused a significant decrease in the number of viable cells compared to vehicle alone (0.1% DMSO). The effects of fucoxanthin on the osteoclastogenesis of RAW264.7 cells

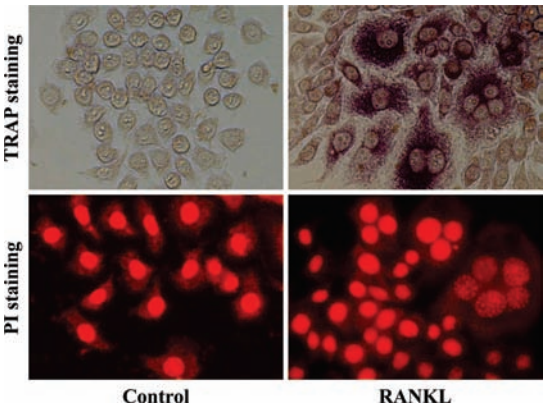

Figure 2. Differentiation of RAW264.7 cells into osteoclast-like cells by RANKL. RAW264.7 cells were cultured with receptor activator of nuclear factor κB ligand (RANKL) or not (control) for 4 days and stained with a tartrate-resistant acid phosphatase (TRAP) staining kit or propidium iodide (PI). Osteoclast-like cells were observed as TRAP-positive and multinucleated cells.

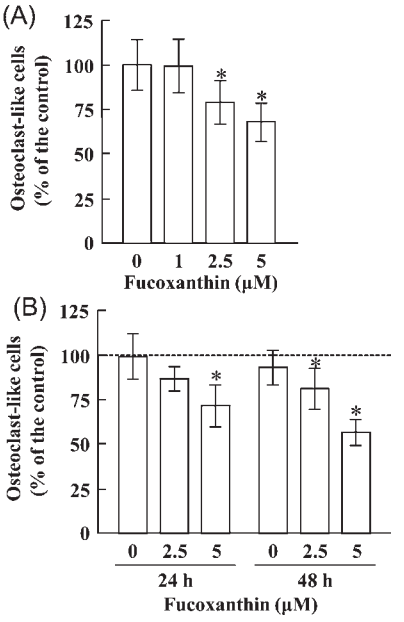

Figure 3. Inhibitory effect of fucoxanthin on the osteoclast differentiation of RAW264.7 cells and the cell viability of osteoclast-like cells: (**A**) RAW264.7 cells were cultured with RANKL and the indicated concentrations of fucoxanthin for 4 days. The number of osteoclast-like cells was counted under a phase-contrast microscope following TRAP staining. The data are representative of three independent experiments and expressed as the mean ± SD of the three experiments (n = 5 culture dishes); *, $p < 0.05$ versus fucoxanthin-untreated control cells (0 μM). (**B**) RAW264.7 cells were cultured with RANKL for 4 days and then cultured with the indicated concentrations of fucoxanthin for another 24 or 48 h. The number of osteoclast-like cells was counted under a phase-contrast microscope following TRAP staining. The values are expressed as the percentage of the cell number before treatment with fucoxanthin. The data are representative of three independent experiments and expressed as the mean ± SD of the three experiments (n = 5 culture dishes); *, $p < 0.05$ versus the values before treatment with fucoxanthin (100%).

were evaluated at concentrations of < 5 μM in the following experiments.

Suppressive Effects of Fucoxanthin on RANKL-Induced Osteoclastogenesis. The treatment of RAW264.7 cells with RANKL (50 ng/mL) for 4 days increased the number of TRAP-positive and multinucleated cells compared with control cells untreated with RANKL (**Figure 2**). Therefore, the effects of fucoxanthin on the osteoclastogenesis of RAW264.7 cells were evaluated after treatment with 50 ng/mL RANKL for 4 days. After treatment with RANKL in the absence or presence of fucoxanthin, the TRAP-positive multinucleated cells were counted as osteoclast-like cells. Fucoxanthin at concentrations of > 2.5 μM caused a significant decrease in the number of osteoclast-like cells (**Figure 3A**), suggesting that fucoxanthin inhibited the differentiation of RAW264.7 cells to osteoclast-like cells and/or that fucoxanthin induced cell death in the osteoclast-like cells. To investigate the cytotoxicity of fucoxanthin to osteoclast-like cells, osteoclast-like cells differentiated by RANKL stimulation were further cultured in the absence or presence of fucoxanthin (**Figure 3B**). Fucoxanthin treatment significantly decreased the number of cells by 24 and 48 h after treatment. Therefore, the decreases in the number of osteoclast-like cells shown in **Figure 3A** were due to the cytotoxic effect of fucoxanthin on differentiating and/or differentiated cells.

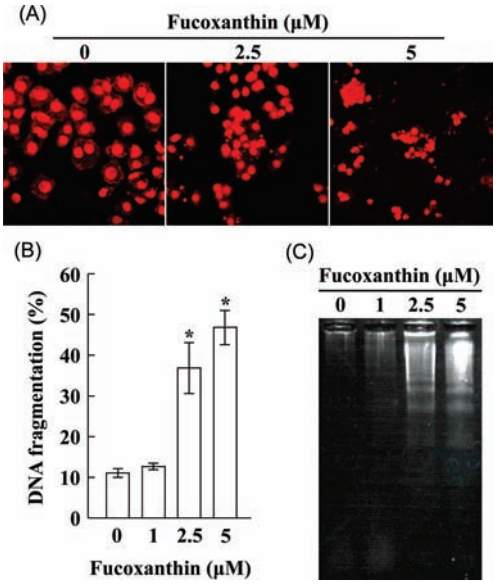

Figure 4. Induction of apoptosis by fucoxanthin in osteoclast-like cells. Osteoclast-like cells were treated with the indicated concentrations of fucoxanthin for 48 h: (**A**) the morphological changes that occurred following PI staining were observed under a fluorescent microscope; (**B**) DNA fragmentation was quantitatively measured using DAPI [values were calculated as a percentage of fragmented DNA in total; data are represented as means ± SD (n = 3 culture dishes; *, $p < 0.05$ versus fucoxanthin-untreated control cells (0 μM)]; (**C**) nucleosomally fragmented DNA was analyzed by 2% agarose gel electrophoresis (data are representative of triplicate experiments).

Apoptosis Induction by Fucoxanthin in Osteoclast-like Cells. To further investigate the cytotoxicity of fucoxanthin to osteoclast-like cells, osteoclast-like cells were treated with fucoxanthin for 48 h and morphologically observed after PI staining. Treatment with fucoxanthin at > 2.5 μM caused cell and nucleus shrinkage, nuclear fragmentation, and the formation of apoptotic bodies, which are characteristic of apoptotic cells (**Figure 4A**). In addition to 48 h treatment, 24 h treatment with fucoxanthin at 5 μM also induced morphological changes but did not do so at 2.5 μM (data not shown). DNA fragmentation is another hallmark of apoptotic cells (*28*). Fucoxanthin increased DNA fragmentation in a dose-dependent manner 48 h after the treatment (**Figure 4B**), and the agarose gel electrophoresis clearly showed the DNA ladder

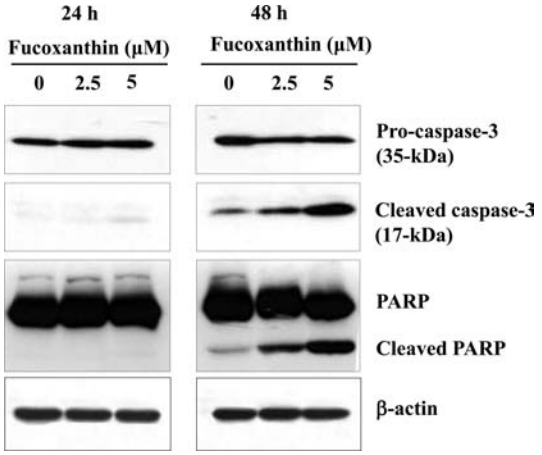

Figure 5. Involvement of caspase-3 activation in fucoxanthin-induced apoptosis of osteoclast-like cells. Osteoclast-like cells were treated with the indicated concentrations of fucoxanthin for 24 or 48 h, and apoptosis-related proteins were analyzed by Western blotting analysis. Equal loading of protein was confirmed by β-actin. The data are representative of duplicate experiments.

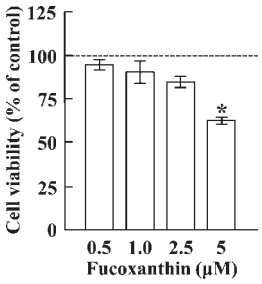

Figure 6. Effect of fucoxanthin on the viability of MC3T3-E1 cells. MC3T3-E1 cells were treated with the indicated concentrations of fucoxanthin for 48 h, and the viable cells were counted by the trypan blue exclusion assay. The values are expressed as the percentage of fucoxanthin-untreated control cells, and the data represent the mean ± SD of three culture dishes; *, $p < 0.05$ versus fucoxanthin-untreated control cells.

pattern characteristic of apoptosis by the treatment with 2.5 and 5 μM fucoxanthin (**Figure 4C**). These results indicate that the cytotoxicity of fucoxanthin in osteoclast-like cells was accompanied by apoptosis induction.

Activation of Caspase-3 in Fucoxanthin-Treated Osteoclast-like Cells. To investigate the mechanism of fucoxanthin-induced apoptosis, the involvement of caspase activation in the apoptosis of osteoclast-like cells was examined at 24 and 48 h after treatment with fucoxanthin at 2.5 or 5 μM. Western blotting analysis showed that fucoxanthin treatment significantly increased the amount of the cleaved 17-kDa fragment of caspase-3, the active form, and decreased the amount of the 35 kDa pro-caspase-3 at 48 h, indicating that fucoxanthin activated caspase-3 (**Figure 5**). In addition, fucoxanthin also caused the cleavage of PARP, an intracellular substrate of caspase-3. These results indicate that fucoxanthin activated caspase-3 and sequentially cleaved PARP, resulting in apoptotic induction.

Cytotoxicity of Fucoxanthin toward Osteoblast MC3T3-E1 Cells. Bone remodeling is continually controlled according to physiological circumstances through bone resorption by osteoclasts and bone formation by osteoblasts (*1*−*3*). To investigate the cytotoxic effects of fucoxanthin on osteoblasts, cells of the osteoblast-like cell line MC3T3-E1 were cultured in several concentrations of fucoxanthin for 4 days (**Figure 6**), and the

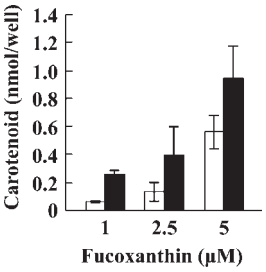

Figure 7. Intracellular fucoxanthin and fucoxanthinol in osteoclast-like cells. Osteoclast-like cells were treated with 1 μM (1 nmol/well), 2.5 μM (2.5 nmol/well), or 5 μM (5 nmol/well) fucoxanthin for 48 h. Cells were harvested, and intracellular fucoxanthin (open bars) and fucoxanthinol (solid bars) were extracted and analyzed by HPLC. The data represent the mean ± SD of three wells.

effects were evaluated according to the same method as shown in **Figure 3A**. Fucoxanthin at 5 μM showed significant cytotoxicity, but did not at concentrations of < 2.5 μM. These results indicate that the cytotoxicity of fucoxanthin against osteoclasts is stronger than that against osteoblasts.

Amount of Fucoxanthin and Fucoxanthinol in Osteoclast-like Cells. To investigate the intracellular concentrations of fucoxanthin and its metabolite fucoxanthinol, osteoclast-like cells were treated with fucoxanthin for 48 h, and the intracellular concentration was analyzed with HPLC. The major peaks completely fitted with the peaks corresponding to the standards for fucoxanthin and fucoxanthinol when the extract mixed with these standards was injected to the HPLC (data not shown). The intracellular fucoxanthin and fucoxanthinol increased in a dose-dependent manner (**Figure 7**). More than 60% of fucoxanthin incorporated into cells was hydrolyzed to fucoxanthinol. This result indicates that fucoxanthinol may play an important role in inducing apoptosis in osteoclast-like cells.

DISCUSSION

In the present study, the effects of fucoxanthin on osteoclastogenesis were investigated using cells of the macrophage cell line RAW264.7, which have the capacity to differentiate into osteoclast-like cells after RANKL stimulation. Fucoxanthin significantly suppressed the differentiation of RAW264.7 cells at 2.5 μM (**Figure 3A**), which was not toxic to RAW264.7 cells (**Figure 1**). Treatment with 2.5 μM fucoxanthin also induced apoptosis via the activation of caspase-3 (**Figures 3B**, **4** and **5**) in osteoclast-like cells. On the other hand, 2.5 μM fucoxanthin did not decrease the cell viability of the osteoblast-like cell line MC3T3-E1 (**Figure 6**), indicating that the apoptosis-inducing activity of fucoxanthin in osteoclasts is greater than that in osteoblasts. The present in vitro study suggested that fucoxanthin at certain concentrations suppresses osteoclastogenesis via the inhibition of osteoclast differentiation and the induction of apoptosis in osteoclasts, but that it does not deleteriously affect bone formation.

β-Cryptoxanthin, a carotenoid present in citrus fruits, has a potent inhibitory effect on osteoclast-like cell formation in mouse marrow cultures stimulated with RANKL, and no inhibition was observed in the presence of an inhibitor of protein synthesis, cycloheximide or 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (*23*). Osteoprotegerin is a regulated suppressor of osteoclast differentiation (*3*) and is excreted from osteoblasts/stromal cells (*32*). Uchiyama et al. (*23*) speculated that the inhibitory effects of β-cryptoxanthin might be due to the inhibition of protein synthesis such as via osteoprotegerin. In the present study, fucoxanthin also inhibited the RANKL-stimulated differentiation of RAW264.7 cells to osteoclast-like cells (**Figure 3A**),

but it is unknown whether RAW264.7 cells have the potency to express and excrete suppressor proteins such as osteoprotegerin. We speculate that the inhibitory effect of fucoxanthin in the present study may be unrelated to osteoprotegerin synthesis. Further studies are needed to verify the effects of fucoxanthin on protein synthesis such as its effects on osteoprotegerin expression.

Fucoxanthin induced apoptosis in osteoclast-like cells derived from RAW264.7 cells (**Figures 3B**, **4**, and **5**). Our previous studies demonstrated that fucoxanthin also induced apoptosis in colon carcinoma WiDr and HCT116 cells (*15*) as well as hepatic carcinoma HepG2 cells (*33*). Furthermore, it caused apoptotic cell death in prostate cancer PC-3, DU145, and LNCaP cells (*34*) and leukemia HL-60 cells (*35*). Thus, fucoxanthin is able to induce apoptosis in several cell lines; however, the effective concentration differed among the cell lines, and the mechanism of its action is unknown. In a study of WiDr cells (*15*), the apoptosis induced by fucoxanthin might have followed cell cycle arrest because fucoxanthin caused cell cycle arrest at the G_0/G_1 phase through the up-regulation of p21 at low concentrations and induced apoptosis at high concentrations. A study of HL-60 cells (*35*) concluded that apoptosis induction by fucoxanthin may be mediated via mitochondrial membrane permeabilization and caspase-3 activation, but not involved with changes in the protein levels of Bcl-2, Bcl-X_L, and Bax. The present study also indicated that fucoxanthin-induced apoptosis was accompanied by the activation of caspase-3 and the cleavage of PARP, a substrate of caspase-3 (**Figure 5**). These results are consistent with the results of a previous study using HL-60 cells (*35*). Probably, the apoptotic mechanism in osteoclast-like cells is similar to that in other cell lines as reported previously. In addition to fucoxanthin, β-cryptoxanthin has also been reported to induce apoptosis in osteoclastic cells formed in a mouse marrow culture system in vitro (*36*, *37*).

The present study demonstrated the inhibitory effect of fucoxanthin on osteoclastogenesis in an in vitro system. However, the effects of fucoxanthin should also be discussed in terms of its bioavailability, and dietary fucoxanthin is considered to be hydrolyzed to fucoxanthinol in the gastrointestinal tract by digestive enzymes such as lipase and cholesterol esterase and then absorbed into intestinal cells (*31*). Recently, we reported the distributions and concentrations of fucoxanthin and its metabolites in mice orally administered fucoxanthin (*38*, *39*). According to these studies, some dietary fucoxanthin was absorbed into the body without being metabolized and was detectable in the liver, lung, kidney, heart, spleen, and adipose tissue of the mice, but the concentrations of fucoxanthinol were higher than the concentrations of fucoxanthin in these tissues. Therefore, the bioavailability of fucoxanthinol is higher than that of fucoxanthin in the body. Furthermore, a previous study using human leukemia HL-60 cells (*40*) indicated that the apoptosis-inducing activity of fucoxanthinol was stronger than that of fucoxanthin. The present study also showed that >60% of the fucoxanthin incorporated into osteoclast-like cells was hydrolyzed to fucoxanthinol, suggesting the active compound to induce apoptosis may be fucoxanthinol. Taken together, most dietary fucoxanthin may be converted to fucoxanthinol, and fucoxanthinol may exert a suppressive effect on osteoclastogenesis at concentrations lower than the effective concentrations of fucoxanthin used in the present study.

Yamaguchi et al. (*41*) investigated the effects of marine algae on bone calcification in the femoral-metaphyseal tissues of rats. In their study, the marine algae were lyophilized and powdered, and rats were orally administered a 5% water suspension of the powder (1.0 mL per 100 g of body weight). Although the administration of a water suspension of *U. pinnatifida* or *E. bicyclis* powder increased calcium content in the femoral-metaphyseal tissues of rats, the bone calcium concentration did not increase in in vitro experiments using water-solubilized extracts from *U. pinnatifida* or *E. bicyclis* powder, indicating that the active compounds in *U. pinnatifida* and *E. bicyclis* are fat-soluble compounds such as carotenoids. Fucoxanthin is a carotenoid produced specifically by edible brown sea algae, and 11.1, 7.7, and 18.7 mg of fucoxanthin are present per 100 g of fresh raw *U. pinnatifida* Suringar, *E. bicyclis* Setch., and *L. japonica* Areschoug sea algae, respectively (*42*). Kanazawa et al. showed that ethanol concentrations of <25% extracted less fucoxanthin from raw *L. japonica* Areschoug, whereas absolute ethanol (99%) gave the highest recovery ratio of fucoxanthin (*42*). This indicates that it is impossible to extract fucoxanthin from *U. pinnatifida* and *E. bicyclis* powder. We speculate that the water-solubilized extracts of *U. pinnatifida* or *E. bicyclis* powder used in the previous study (*41*) contained less fucoxanthin, resulting in the lack of increase in bone calcium observed in their in vitro experiments. Therefore, the fucoxanthin in *U. pinnatifida* and *E. bicyclis* powder might have been the active compound that increased bone calcification in previous in vivo experiments (*41*).

In conclusion, fucoxanthin induced apoptotic cell death in osteoclast-like cells at a concentration nontoxic to osteoblast-like cells. These results indicate that dietary fucoxanthin is helpful for the prevention of bone diseases such as osteoporosis and rheumatoid arthritis, which are known to be related to bone resorption.

ABBREVIATIONS USED

DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; PI, propidium iodide; RANKL, receptor activator of nuclear factor κB ligand; TRAP, tartrate-resistant acid phosphatase.

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