CHAPTER 34

Extracts of Marine Algae Show Inhibitory Activity Against Osteoclast Differentiation

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Contents

Abstract

Osteoclasts are multinucleated cells that play a crucial role in bone resorption. The imbalance between bone resorption and bone formation results in osteoporosis. Therefore, substances that can suppress osteoclast formation are potential candidate materials for drug development or functional foods. There have been reports that extracts or purified compounds from marine micro- and macroalgae can suppress osteoclast differentiation. Symbioimine, isolated from the cultured dinoflagellate *Symbiodinium* sp., had suppressive effects against osteoclast differentiation in osteoclast-like cells. Norzoanthamine, isolated from the colonial zoanthid *Zoanthas* sp., has been shown to have antiosteoporosis activity in ovariectomized mice. With regard to marine extracts, the fucoxanthin-rich component from brown algae has been shown to have suppressive effects against osteoclast differentiation. An extract of

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Sargassum fusiforme has recently been shown to have antiosteoporosis activity. This extract suppressed both osteoclast differentiation and accelerated osteoblast formation in separate *in vitro* experiments. It also showed antiosteoporosis activity in ovariectomized mice by regulating the balance between bone resorption and bone formation. These marine algae and their extracts may be sources of marine medicinal foods for the prevention of osteoporosis.

I. INTRODUCTION

Osteoporosis is a disabling disorder that is characterized by decreased bone strength, which predisposes patients to an increased risk of bone fracture. Peak bone mass (maximum bone strength and density) is normally attained by age 20–25. Age, race, sex, environment, and lifestyle factors such as physical activity and diet are important determinants of bone density. With the increase in age, the rate at which bone tissue is replaced is reduced in comparison to the rate at which it is lost, which increases the risk for fractures.

As bone loss is a gradual and painless process, osteoporosis is often diagnosed only after the occurrence of a fracture, which makes it challenging to reliably estimate the patient population that is at risk for developing this disease. Worldwide, 200 million people are estimated to suffer from osteoporosis (Cooper, 1999). While the overall number of fractures is increasing worldwide according to the report from World Health Organization, several reports from population-based studies have shown that recent progress in the diagnosis and treatment of osteoporosis has been effective at reducing the number of hip fractures (Jaglal *et al.*, 2005).

Pharmacological and nutritional factors may help to prevent bone loss with increasing age. Nutritional factors may be especially important in the prevention of osteoporosis (Bonjour *et al.*, 1996; Yamaguchi, 2002, 2006). While chemical factors in food and plants may also help to prevent bone loss with increasing age, these factors are poorly understood.

There has been a major increase in lifestyle-related diseases due to the fact that we are living longer and have adopted a Western diet.

In Japan, osteoporosis is currently treated with active form of vitamin D_3 (Orimo *et al.*, 1994; Schacht, 1999), estrogen (Lufkin *et al.*, 1992; Weinstein *et al.*, 2003), calcitonin (Body, 2002; Kopaliani, 2005), ipriflavone (Nakamura *et al.*, 1992), vitamin K_2 (Sakamoto *et al.*, 2005; Steven *et al.*, 2005), bisphosphonate (Hamdy *et al.*, 2005; Matsumoto, 2004; Perez-Lopez, 2004), and related compounds. However, although these drugs are used to treat osteoporosis, they cannot prevent it. In addition, some of

these drugs, such as calcitonin, are not easily administered, and the emergence of drug resistance has been observed. The active form of vitamin D_3 is not effective for treating hypercalcemia, and bisphosphonate inhibits bone formation. The use of estrogen should be careful, while estrogen administration for even just 6 months has been observed to result in abdominal bloating, breast pain, digestive symptoms, and irregular vaginal bleeding (Barkhem *et al*., 1998). These limitations of medications suggest that the prevention of osteoporosis through diet is very important.

Bone health and the prevention of osteoporosis-related fractures are key elements in the strategy for managing patients undergoing menopause. A detailed knowledge of bone health and related diagnostic and therapeutic options falls within the domain of the gynecologist as part of a multidisciplinary approach.

Osteoporosis-related fractures are common and will affect at least one-third of women over 50 years of age (Johnell and Kanis, 2005). It is estimated that osteoporosis affects 75 million people in Europe, the United States, and Japan, and this is estimated to increase by 240% by 2050.

Bone metabolism is characterized by two opposing activities: bone formation and bone resorption (Martin, 2002). Once formed, the bones in adults are continuously remodeled, and the remodeling rate is between 2% and 10% of the skeletal mass per year. Bone mass depends on the balance between resorption and formation within the remodeling unit. As osteoporosis is characterized by a decrease in bone mass with deterioration in the architecture of bones, it is the result of an imbalance between bone formation and resorption.

Bone remodeling is disturbed under a variety of pathologic conditions that affect the skeleton, including postmenopausal osteoporosis and rheumatoid arthritis, in which there is a local and/or systemic alteration in the levels of hormones or proinflammatory cytokines that are known to stimulate or inhibit bone resorption *in vitro* and *in vivo*. Parathyroid hormone has been recognized as a stimulator of bone resorption since the early 1980s (Rodan and Martin, 1981). Studies with genetically altered mice and other animal models of bone diseases over the past 10 years have greatly increased our knowledge of factors that regulate the formation and activity of osteoclasts. In particular, identification of the receptor activator of nuclear factor-κB ligand (RANKL)/RANK/osteoprotegerin signaling system in the mid- to late-1990s represented a major breakthrough that clarified the role played by osteoblasts in this process. Moreover, it has become increasingly clear that osteoclasts are not simply trench-digging cells; instead, they have important regulatory functions as immunomodulators in pathologic states and also help to control osteoblast function (Martin and Slims, 2005).

II. METHODS FOR ASSAYING OSTEOBLASTS AND OSTEOCLASTS *IN VITRO*

In general, these activities have been evaluated using an experimental system, which leads to the differentiation of mature osteoclasts, in which bone marrow cells are cultured in the presence of osteoblasts.

Bone remodeling is a continuous process that helps to repair the microdamage to the bone matrix and adjusts the bone architecture to maintain bone strength. In this tightly regulated process, osteoclasts, which are multinucleated cells derived from the myeloid hemopoietic lineage, comprise the principal cell population that is involved in bone resorption, whereas osteoblasts, which originate from multipotent mesenchymal stem cells, carry out bone formation. The overall integrity of bone is controlled by biochemical factors, which include hormones, cytokines and other proteins, and mechanical factors. Perturbations of this complex but well-coordinated process result in skeletal abnormalities characterized by increased bone loss or excessive bone formation.

Osteoclast-like multinucleated cells can be differentiated *in vitro* from cocultures of mouse bone marrow cells and calvarial osteoblastic cells by treatment with the osteotropic factor 1α,25-dihydroxyvitamin D_3 (1,25 $(OH)_2D_3$) (Miura *et al.*, 2002). It has been shown that RANKL induces osteoclast formation in a culture of bone marrow cells in the presence of macrophage colony-stimulating factor without the need for osteoblasts. A mouse macrophage RAW264 cells are also known to differentiate into osteoclasts in the presence of RANKL (Hsu *et al.*, 1999). When osteoblast/stromal cells are stimulated by osteotropic factors such as $1,25(OH)_2D_3$, RANKL is expressed and induces the differentiation of osteoclast progenitors by binding to RANK (Jimi *et al.*, 1999).

Osteoblasts were isolated from 3-week-old mice that had been killed by cervical dislocation. The calvariae were digested in 2 ml of an enzyme solution containing 0.2% collagenase (Wako, Osaka, Japan) for 5 min at 37 °C in a shaking water bath. The supernatant was discarded and 2 ml of the enzyme solution was added. After the mixture was shaken at 37 °C for 20 min, the supernatant was carefully collected and transferred to a new tube. This digestion of calvariae by collagenase was repeated three times. The collected supernatant (6 ml) was placed in a centrifuge at 1500 × *g* for 5 min to collect osteoblastic cells. Cells were resuspended in α-minimal essential medium (α-MEM) (MP Biomedicals, Germany) containing 10% fetal bovine serum (FBS) and cultured to confluence in culture dishes for about 1 week. The cells were then detached from the culture dishes using trypsin–EDTA, suspended in α-MEM containing 10% FBS, and used for the coculture as osteoblastic cells.

Femoral and tibial bone marrow cells were collected from 7-week-old mice that had been killed by cervical dislocation. The tibiae and femora

were removed and dissected free of adhering tissues. The bone ends were removed, and the marrow cavities were flushed by slowly injecting media at one end using a 26-gauge needle. The calvariae and bone marrow cells were washed and used in the coculture.

Mouse calvarial cells (1.3×10^5 cells/ml) were cocultured with bone marrow cells (5.0×10^6 cells/ml) in α-MEM containing 10% FBS in 48-well plates (Corning Inc., NY, USA). The culture volume was made up to 250 μl per well with α-MEM supplemented with 10% FBS, in the presence of 10 ng/ml $1,25(OH)_2D_3$ (Biomol, PA, USA), with or without samples. All cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 in air. Half of the medium was changed after coculture for 3 days.

To count multinucleated cells, cultured cells were stained as described below. After cells were cultured, adherent cells were fixed with 10% formaldehyde in phosphate-buffered saline (−) for 20 min. After cells were treated with 95% ethanol for 1 min, the well surface was dried and treated with tartrate-resistant acid phosphatase (TRAP)-staining solution [0.1 M sodium acetate buffer (pH 5.0) containing 50 mM sodium tartrate, 0.1 mg/ml naphthol AS-MX phosphate (Sigma Chemical Co., St. Louis, USA), and 1 mg/ml fast red violet LB salt (Sigma Chemical Co.)] for 30 min. TRAP-(+) multinucleated cells were then counted under a microscope.

Cell viability was evaluated using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) (Sigma Chemical Co.) assay. After culture, cells were treated with 1 mg/ml MTT for 2 h, precipitated dye was solubilized in dimethylsulfoxide, and the absorbance at 570 nm was measured.

III. MICROALGAE

The symbiotic microalga *Symbiodinium* sp., which is a type of symbiotic zooxanthellae, is found in a wide range of marine invertebrates. Dinoflagellates are widely known to be a rich source of biologically active and structurally unique secondary metabolites (Kita and Uemura, 2005, 2006; Uemura, 2006). Some dinoflagellates were cultured under artificial conditions with seawater medium, and the alga body was then centrifuged and extracted with 80% aqueous EtOH to collect metabolites. Symbioimines (Kita *et al.*, 2004, 2005), symbiodinolide (Kita *et al.*, 2007), and symbiospirols (Tsunematsu *et al.*, 2009) have been isolated from the same strain of *Symbiodinium* sp. derived from the marine acoel flatworm. One of these compounds, symbioimine (Fig. 34.1), an amphoteric iminium metabolite, has been shown to be an antiresorptive and anti-inflammatory drug (Kita *et al.*, 2005). Its ability to suppress osteoclast differentiation ($EC_{50} = 44$ μM) was demonstrated in RAW264 cells. In addition, symbioimine (10 μM) also inhibited cyclooxygenase-1 and -2 activities by 5% and 32%, respectively.

The zoanthamine alkaloids are a structurally unique family of natural products that exhibit antiosteoporotic, antibiotic, anti-inflammatory, and cytotoxic biological activities. Although they are isolated from soft coral of the order zoantharia, symbiotic algae may play an important role in their biosynthesis. Norzoanthamine (Fig. 34.2) was isolated along with some analogs from a *Zoanthus* species collected off the Ayamaru coast of the Amami Islands in Japan (Fukuzawa *et al.*, 1995). Norzoanthamine and its hydrochloride salt have been shown to prevent bone loss in ovariectomized mice, a pharmaceutical model for postmenopausal osteoporosis (Kuramoto *et al.*, 1996). As ovariectomized mice do not produce sufficient estrogen, they quickly lose bone mass and strength within a few weeks. However, oral treatment of mice with norzoanthamine HCl at doses of

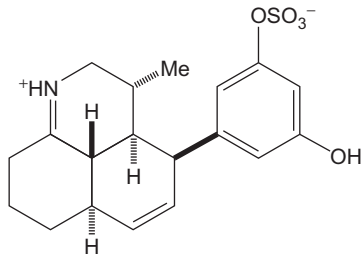

FIGURE 34.1 Structure of symbioimine. Symbioimine, an amphoteric iminium compound, was isolated from symbiotic algae of the marine acoel flatworm *Amphiscolops* sp. which was collected at Sesoko Island, Okinawa, Japan.

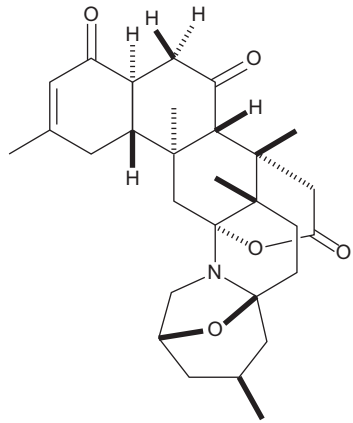

FIGURE 34.2 Structure of norzoanthamine. Norzoanthamine, an antiosteoporotic marine alkaloid, was isolated along with some analogs from a *Zoanthus* species collected off the Ayamaru coast of the Amami Islands in Japan.

0.08–2.0 mg/kg/day for 4 weeks led to a significantly higher retention of femur weight than in the control group (Kuramoto *et al.*, 1996, 1998; Uemura, 2006). Further, these preventive effects were not accompanied by an increase in uterine weight, which is a serious side effect of treatment with 17b-estradiol (Yamaguchi *et al.*, 1999). *In vitro* studies with norzoanthamine showed that it had no effect on the formation of osteoclasts, and the suppression of IL-6 secretion, which has been suggested by *in vitro* experiments (Kuramoto *et al.*, 2000), has not yet been demonstrated *in vivo* (Behenna *et al.*, 2008). Further investigations will be needed to elucidate the mechanism of the antiosteoporosis action of norzoanthamine.

IV. MACROALGAE

The suppressive effects of macroalgae extract against osteoclast differentiation have been reported as described below.

Uchiyama *et al.* (2004) investigated the effect of the water-soluble extract from marine algae *Sargassum horneri* on osteoclastic bone resorption and osteoblastic bone formation *in vitro*. They found two components in the crude extract: heat-labile component with a molecular weight of 1000 and heat-stable component with a molecular weight of 50,000. The former component increased the calcium content in rat femoral-diaphyseal tissues at 25 μg/ml (Uchiyama and Yamaguchi, 2003; Yamaguchi *et al.*, 2001), and the latter suppressed $1,25(OH)_2VD_3$-induced osteoclast-like cell formation. Their research group also reported that the *S. horneri* extract helped to prevent bone loss in streptozotocin-induced diabetic rats *in vivo* (Uchiyama and Yamaguchi, 2003). Interestingly, these two active components in *S. horneri* extract were thought to regulate bone metabolism to prevent osteoporosis.

Brown sea algae contain the characteristic carotenoid fucoxanthin. Dietary fucoxanthin has been shown to exhibit various beneficial effects. Das *et al.* (2010) reported that fucoxanthin from edible brown algae significantly suppressed the differentiation of RAW 264.7 cells at 2.5 μM, without any cytotoxic effects. They concluded that fucoxanthin is helpful for the prevention of diseases associated with abnormal bone metabolism, as fucoxanthin induced apoptotic cell death in osteoclast-like cells at a concentration that was nontoxic to osteoblast-like cells. Using the same bioassay system, our research group found that the methanol extract of *Sargassum fusiforme* (SME) suppressed osteoclast differentiation and accelerated osteoblast differentiation. Osteoclast differentiation was estimated by TRAP-(+) multinucleated cell formation in osteoblastic cell/bone marrow cell coculture. Osteoclast formation was induced by the presence of $1,25(OH)_2D_3$ in the coculture. As shown in Fig. 34.3, SME

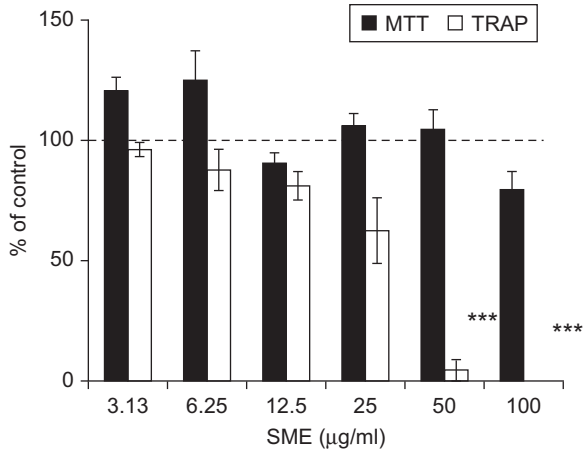

FIGURE 34.3 Effect of methanolic extract of *S. fusiforme* (SME) on osteoclast formation. Closed and open columns indicate cell viability and osteoclast formation, respectively. TRAP-positive multinucleated cells that had more than three nuclei were counted. Cell viability was determined by MTT assay. Data are expressed as the mean ± SE of four cultures. ***$p < 0.005$ versus control using Student's *t*-test.

reduced the number of multinucleated osteoclast cells in a dose-dependent manner without any cytotoxic effects. The addition of SME reduced the number of TRAP-(+) multinucleated cells, without cytotoxicity, to 60% at 25 μg/ml and 6% at 50 μg/ml (Fig. 34.3).

The effects of SME on osteoblast cells were also investigated *in vitro* as shown in Fig. 34.4. Osteoblasts are differentiated from stem cells by their own mediators, alkaline phosphatase (ALP), osteopontin, and osteocalcin. Eventually, mature osteoblasts become calcified to form bone tissue. The addition of bone morphogenetic protein 2 accelerated the differentiation of osteoblasts in a dose-dependent manner at 25–100 ng/ml. On the other hand, SME increased ALP activity, which accelerated the differentiation of osteoblasts in a dose-dependent manner.

The effect of SME on bone resorption *in vivo* was examined in ovariectomized mice, an experimental model of postmenopausal osteoporosis (Masuda *et al.*, 2008). Femurs were isolated from mice at 4 weeks after ovariectomy. The amount of cancellous bone was measured on photographs of the ground bone. The average length of the cancellous bone area on ground sections of femurs in the sham-operated group (5.4 mm) was much greater than that in the ovariectomized group (2.7 mm). The weight loss and decrease in cancellous bone area in the ovariectomized group were likely due to bone resorption enhanced by estrogen deficiency. However, oral administration of SEM at 500 mg/kg/day for 4 weeks prevented this decrease, as shown in Fig. 34.5.

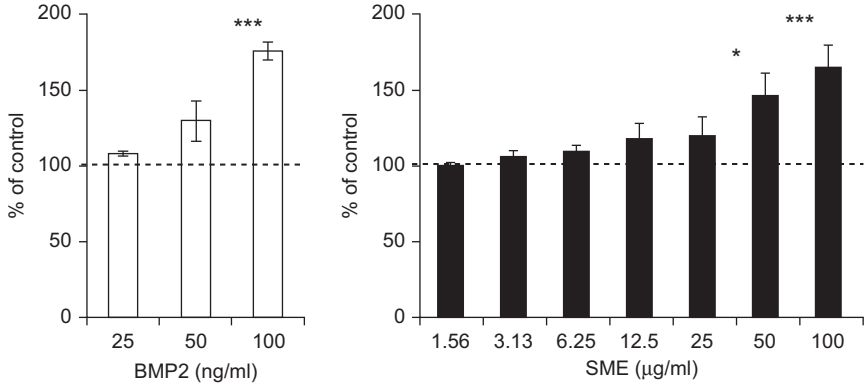

FIGURE 34.4 Effects of methanolic extract of *S. fusiforme* (SME) on osteoblast differentiation *in vitro*. Osteoblasts are differentiated from stem cells by their own mediators *in vitro*. Differentiation rates were evaluated by measuring alkaline phosphatase (ALP) in comparison with control cells without samples. Bone morphogenetic protein 2 (BMP2) was used as a positive control to accelerate the differentiation of osteoblasts. Data are expressed as the mean ± SE of four cultures. *$p < 0.05$ and ***$p < 0.005$ versus control.

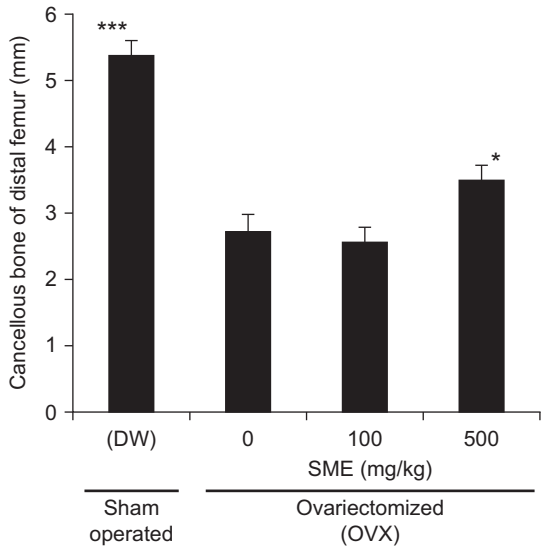

FIGURE 34.5 Effects of methanolic extract of *S. fusiforme* (SME) on bone loss in ovariectomized mice. Four-week-old female ddY mice were subjected to ovariectomy or sham operation under anesthesia. Oral administration of the vehicle or SME dissolved in distilled water (DW) was started from the day after surgery and continued for 5 days per week for 4 weeks. After 4 weeks, mouse body weight was measured and the animals were sacrificed to retrieve their femurs and uterine weight. Dry weight was measured using the right femur after drying at 60 °C for 24 h. Cancellous bone of the distal femur was measured on a photograph of the ground femur. Data are expressed as the mean ± SE of four cultures. *$p < 0.05$ and ***$p < 0.005$ versus control (SME = 0 mg/kg).

SME both promoted osteoblast differentiation and inhibited osteoclast differentiation *in vitro*. Its ability to suppress bone loss has also been demonstrated *in vivo*. SME has been suggested to regulate bone turnover by influencing both osteoblasts and osteoclasts. These two effects are thought to involve other compounds. As these effects were associated with the methanol extract, the active components are thought to be nonpolar, low-molecular-weight molecules. It is expected that the active components will be identified in the near future.

V. CONCLUSION

Extracts and purified compounds from various marine algae have been reported to suppress osteoclast differentiation. Further studies to elucidate the detailed mechanisms and the responsible components may hopefully show that these marine algae are a potential source of marine medicinal foods to prevent osteoporosis and related diseases.

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