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Original article

Shark protein improves bone mineral density in ovariectomized rats and inhibits osteoclast differentiation

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ABSTRACT

Objectives: Fish proteins are potential sources of natural drugs and food additives. There are many studies being performed to develop underutilized fish proteins. Therefore, the aim of this study was to determine how shark protein functions as a dietary supplement for bone health.
Methods: Three groups of ovariectomized (OVX) rats were fed different diets containing 20% casein protein, 20% shark protein, or 20% cod protein for 4 wk. Bone mineral density of the right femur was measured by dual-energy x-ray absorptiometry and quantitative computed tomography. Furthermore, we prepared low-molecular-weight peptides from shark protein using protease for in vitro studies. Calcitriol was added to bone marrow cells and the receptor activator of the nuclear factor-κB ligand was added to RAW264 cells. After 7 d, the number of tartrate-resistant acid phosphatase-positive cells was counted.
Results: In the shark protein-fed group, bone mineral density of the femur epiphysis was higher than that of the casein protein-fed group. In particular, the shark protein-fed group showed an increase in bone mineral density of that was represented mainly by trabecular bone. Shark protein hydrolysates inhibited osteoclast formation in bone marrow cells and RAW264 cells.
Conclusions: These results suggest that shark protein might suppress the bone loss caused by estrogen deficiency through the suppression of osteoclast formation.

Introduction

Shark protein, which is available on the world market, usually comes from sharks that are caught during tuna trawler fishing. The commercial value of the shark is represented mainly by its fin, which is used as a luxury foodstuff. Therefore, shark fins are normally obtained by the controversial practice of finning, in which a shark's fin is cut off and the rest of its body is tossed into the ocean. However, in Japan, the entire shark body is utilized. Its cartilage is used as a source of chondroitin sulfate and collagen is purified from its skin. Although shark protein is the main ingredient in fish-paste products, its commercial value remains low. In our laboratory, we examined how shark protein can be used to increase its commercial value.

Bone is an important organ that regulates mineral homeostasis. Menopause causes physiological changes that can lead to an imbalance between bone formation and bone resorption, resulting in net bone loss and osteoporosis, mainly caused by estrogen deficiency [1,2]. Current therapies for osteoporosis include estrogen replacement therapies and the use of bisphosphonates. These therapies are effective in preventing bone loss caused by menopause, but some are accompanied by adverse side effects, such as uterine bleeding, carcinogenesis, and cardiovascular disease [3–5]. Therefore, diet therapy and lifestyle changes that minimize bone loss in postmenopausal women would be very helpful in decreasing the need for drug therapy to prevent osteoporosis. Recently, hydrolyzed collagen of porcine origin [6], oil palm leaf extract [7], soy isoflavones plus vitamin D_3 [8], and lactoferrin [9] have become available as food supplements for improving bone mineral density (BMD). However, few studies have examined fish protein–derived food factors that have a beneficial effect on BMD. Therefore, the aim of this study was to determine how shark protein functions as a dietary supplement for bone health.

KU performed the experiment and drafted the manuscript. AT and MW advised on the experimental design. YN offered instruction and advice on the manuscript.

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Materials and methods

Materials

Seventeen-wk-old female Wistar rats were purchased from the Sankyo Labo Service Corporation, Inc. (Tokyo, Japan). Casein was purchased from the Oriental Yeast Co., Ltd. (Tokyo, Japan). Alcalase®2.4 L FG was purchased from Novozymes A/S (Bagsvaerd, Denmark). Umamizyme G was purchased from Amano Enzyme Inc. (Aichi, Japan). Murine macrophage RAW 264 cells were provided by RIKEN BRC through the National Bio-Resource Project of the MEXT (Ibaraki, Japan). Fetal bovine serum (FBS), penicillin–streptomycin–neomycin (PSN) antibiotic mixture (100 X), non-essential amino acids, and minimum essential medium (MEM) were purchased from Life Technologies Japan Ltd. (Tokyo, Japan). Receptor activator of nuclear factor-κB ligand from mouse (RANKL), 17 β-estradiol (E2), calcitriol (1 α,25-dihydroxy vitamin D_3 [1,25(OH)2 D3]), and α-MEM were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Tartrate-resistant acid phosphatase (TRAP) staining kit was purchased from Primary Cell Co., Ltd. (Hokkaido, Japan). Other chemical reagents were all of special grade from Wako Pure Chemical Industries, Ltd. (Osaka, Japan)

Preparation of shark protein hydrolysates

Shark protein hydrolysates (SH) were made from fresh great blue shark (*Prionace glauca*) protein. Shark protein was heated in 80°C water for 3 min, and then compressed to remove excess water. The same quantity of distilled water was added along with proteases (Alcalase®2.4 L FG) at 3.333% (dry weight). After 30 min, the protein was further treated with 0.5% (dry weight) proteases (umamizyme G) at 50°C and pH 7.0 for 24 h. Alcalase®2.4 L FG is an exo-type peptidase and umamizyme G is a mixture of endo- and exo-type peptidases with a broad substrate specificity. The reaction was stopped by heating at 85°C for 20 min. After all hydrolysates were centrifuged to remove the precipitates, the supernatant was collected and freeze-dried. To compare with SH, cod protein hydrolysates (CH) also were prepared in the same manner.

Amino acid analysis of casein, shark protein, and cod protein using high-performance liquid chromatography

To determine the amino acid profile of casein, shark protein, and cod protein, reverse-phase chromatography was used. Amino acid analysis was performed using a previously described method [10], with slight modifications [11]. The resulting phenylthiocarbamyl amino acids were separated using an octadecylsilane column (TSKgel ODS-80 TsQA 4.6 mm × 150 mm; Tosoh Corp., Tokyo, Japan) at 1.0 mL/min using a binary linear multistep solvent gradient. Solution A consisted of 50 mM sodium acetate buffer, pH 6.0, containing 3% acetonitrile. Solution B consisted of 60% acetonitrile. Gradient profile was: 0 to 15 min, 0 to 70% B; 15 to 25 min, 70 to 100% B; 25 to 26 min, 100% B; 26 to 28 min, 0% B. The column was maintained at 40°C. Elution peaks were monitored at 254 nm.

Molecular weight distribution of SH and CH by high-performance liquid chromatography

The average-molecular-weight distributions of SH and CH were analyzed by gel-filtration chromatography. Ten mg of two types of peptide sample were dissolved in a mixture of 500 μl of ultrapure water and 500 μl of 45% acetonitrile in water, in the presence of 0.1% trifluoroacetic acid. After solubilization, the solutions were filtered and 50 μl injected into a silica-based column (TSKgel G2500 PWXL 7.8 mm × 30 mm; Tosoh Corp.) using a high-performance liquid chromatography LC-8020 Model II chromatograph (Tosoh Corp.) and elution was performed with 45% acetonitrile in water in the presence of 0.1% trifluoroacetic acid at a flow rate of 0.5 mL/min. The column was maintained at 40°C. The elution peaks were monitored at 215 nm.

Table 1
Composition of the diets*

	Casein†	Shark‡	Cod§
Casein	20	0	0
Shark meat	0	20	0
Cod meat	0	0	20
α-cornstarch	13.2	13.2	13.2
β-cornstarch	40.0486	40.0486	40.0486
Sucrose	10	10	10
Soybean oil	7	7	7
Cellulose powder	5	5	5
Mineral mix (93G-MX)	3.5	3.5	3.5
Vitamin mix (93-VX)	1	1	1
Choline bitartrate	0.25	0.25	0.25
tert-Buthylhydroquinone	0.0014	0.0014	0.0014
Total (%)	100	100	100

* Animals were fed a modified AIN-93 G diet.
† 20% casein diet.
‡ 20% shark protein diet.
§ 20% cod protein diet.

Table 2
Amino acid concentrations in casein, shark protein, and cod protein

Amino acid weight ratio (%)	Casein	Shark protein	Cod protein
Asparitic acid/asparagine	8.2	10.4	11.9
Glutamic acid/glutamine	24.3	16.3	17.4
Hydroxyproline	0.0	0.3	0.2
Serine	4.6	3.6	4.5
Glycine	1.8	5.4	4.5
Histidine	3.2	2.9	2.4
Arginine	3.7	7.0	6.8
Threonine	4.3	5.2	5.1
Alanine	3.1	7.8	6.5
Proline	6.8	2.8	3.0
Tyrosine	5.3	3.0	3.9
Valine	7.0	5.4	5.1
Methonine	1.8	3.7	3.7
Cysteine	0.2	0.5	0.3
Isoleucine	4.5	5.3	4.0
Leucine	8.7	9.0	7.7
Hydroxylysine	0.0	0.0	0.0
Phenylalanine	5.1	4.1	3.8
Lysine	7.5	7.2	9.2

Animals and diets

The study was conducted in accordance with the guidelines of the Committee for Animal Research at Tokyo University of Agriculture and Technology. Seventeen-wk-old female Wistar rats (N = 28) were given a modified AIN-93 G diet containing 20% casein for 3 d of acclimatization ad libitum. The rats were housed in individual cages at 22 ± 1°C, 50 ± 5% humidity, on a 12-h light–12-h dark cycle, with water ad libitum. On day 7 of the experiment, 22 rats were ovariectomized (OVX) and 6 rats were sham-operated (sham). OVX rats were then divided into three groups of six or eight rats. They were fed ad libitum for 4 wk on a diet consisting of AIN-93 G with 200 g/kg casein protein as the protein source; the casein being replaced with shark protein (OVX shark) or cod protein (OVX cod); the composition of this diet is presented in Table 1. Weight was recorded every 4 d, and food intake measured every second day of the experiment. After sacrifice, the right femur was excised from each rat to determine bone density after all muscles and connective tissues had been removed.

Measurement of BMD dual-energy X-ray absorptiometry

The right femur was dissected and preserved in 70% ethanol at 4°C. BMD was measured by dual-energy x-ray absorptiometry with a Dichrom Scan PCS-600 instrument (Hitachi Aloka Medical, Ltd., Tokyo, Japan), starting scans in the most proximal area and ending in the most distal. During data analysis, the femur was divided into 20 equal segments along its major axis [12,13].

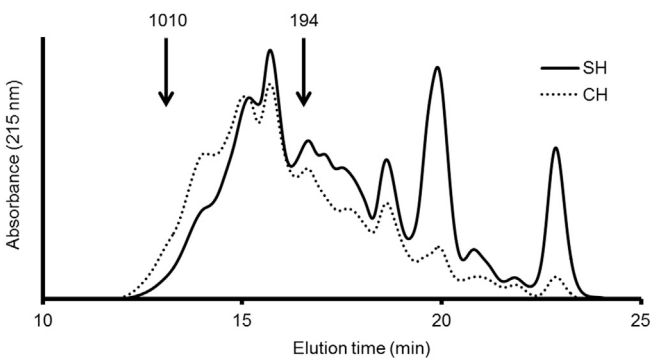

Fig. 1. Elution pattern of shark protein hydrolysates (SH) and cod protein hydrolysates (CH) by gel-filtration chromatography. SH prepared from shark protein and CH prepared from cod protein were analyzed using a silica-based column. Elution was monitored by absorbance at 215 nm. Arrows indicate elution positions of standard molecular weight.

Table 3
Effect of shark protein and cod protein on several parameters*

	Sham	OVX		
	Casein (n = 6)	Casein (n = 8)	Shark (n = 6)	Cod (n = 8)
Final body weight (g)	262.3 ± 9.3†	295.4 ± 23.1	295.4 ± 20.0	291.8 ± 15.3
Body weight gain (g)	19.9 ± 5.9†	44.9 ± 8.2	42.6 ± 12.6	41.3 ± 8.3
Food intake (g/d)	13.9 ± 1.0†	17.1 ± 1.4	17.0 ± 1.2	17.0 ± 1.3
Uterine weight (g)	0.66 ± 0.11†	0.13 ± 0.04	0.13 ± 0.02	0.12 ± 0.09
Femur length (mm)	35.67 ± 0.35	35.92 ± 0.99	35.87 ± 0.59	35.97 ± 0.69
Femur weight (g)	0.77 ± 0.01	0.73 ± 0.05	0.76 ± 0.05	0.75 ± 0.02

OVX, ovariectomized rats; Sham, sham-operated rats
* Data are shown as means ± SD (n = 6 or 8).
† Significant difference from OVX casein group at $P < 0.01$.

Measurement of BMD by quantitative computed tomography

Total BMD, cortical BMD, and trabecular BMD of the right femur were measured by quantitative computed tomography (QCT) using a LaTheta LCT-100 (Hitachi Aloka Medical, Ltd.) with cross-sectional images of 480 × 480 pixels and a slice thickness of 1 mm. The tube voltage of the x-ray generator was 50 kV (1 mA). BMD was evaluated for the proximal, middle, and distal parts of the femur by dividing the femur into three equal lengths. The scans were analyzed with LaTheta software (version 1.40).

Cell culture

Femoral bone marrow cells were collected from female Wistar rats (weight: About 240 g). Femurs were removed and dissected free of adhering tissues. Bone ends were removed and marrow cavities flushed by slowly injecting media at one end using a 23-gauge needle. Bone marrow cells (1.0 × 106 cells/mL) were cultured in α-MEM containing 10% FBS and 1% PSN antibiotic mixture in 48-well plates. The culture volume was 500 μμL per well. In addition to the presence of 1,25(OH)2 D_3 (10-8 M), E2 (10-8 M), or SH (10 μg/mL) was added for 6 d. All cultures were maintained at 37°C in a humidified atmosphere containing 5% carbon dioxide in air. One-half of the medium was replaced every second d.

Murine RAW264 cells were grown in MEM supplemented with 10% FBS, 1% PSN antibiotic mixture, and 0.1 mM non-essential amino acids. For osteoclast differentiation, RAW264 cells (8 × 102) in 0.2 mL were seeded into wells of a 96-well plate in α-MEM supplemented with 10% FBS and 1% PSN antibiotic mixture, and cultured overnight. They were then further incubated for 7 d in the presence of RANKL (50 ng/mL) and E2 (10-8 M), with SH (10 μg/mL) or CH (10 μg/mL). All cultures were maintained at 37°C in a humidified atmosphere containing 5% carbon dioxide in air. One-half of the medium was replaced every second d.

Osteoclast formation was evaluated by counting the number of TRAP-positive stained cells.

Osteoclast differentiation assay

Osteoclast formation was measured by quantifying the cells positively stained by TRAP. Briefly, the cells were fixed using 10% formalin neutral buffer solution for 5 min and then stained using a TRAP staining kit according to the manufacturer's instructions. TRAP-positive staining cells with more than three nuclei were counted as TRAP-positive staining multinucleated cells using light microscopy.

Statistical analyses

Data were expressed as means and SD for the number of measurements shown in the figures or tables. Statistical analysis was carried out by analysis of variance, using Excel 2007 (Microsoft, Redmond, WA, USA) with the add-in software Statcel 3 (OMS Publishing Inc, Saitama, Japan). The significance of differences was determined using Dunnett's multiple comparison test. The probability level used to determine statistical significance was $P < 0.05$.

Results

Properties of casein, shark protein, and cod protein

Casein, shark protein, and cod protein were analyzed for amino acid composition, as shown in Table 2. Shark protein was

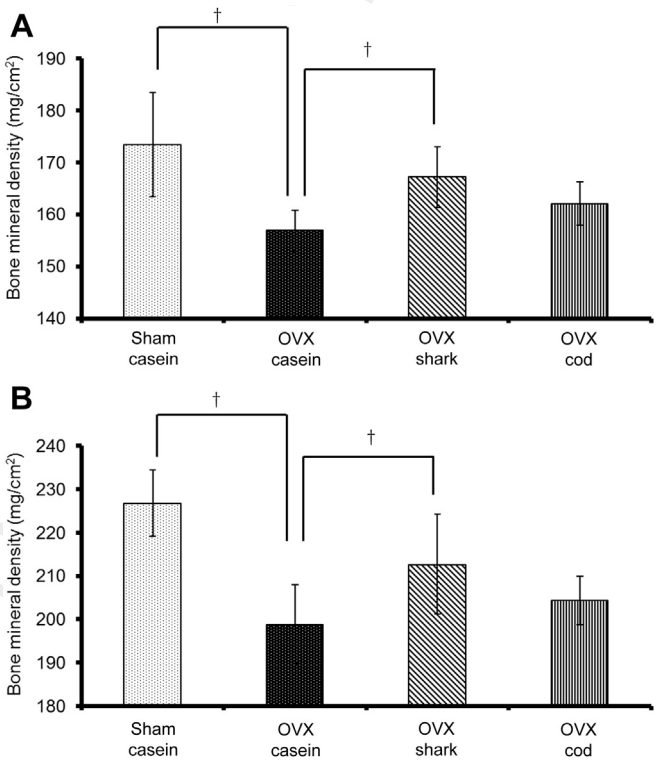

Fig. 2. Femur mineral density by dual-energy x-ray absorptiometry (DXA). Bone mineral density (BMD) levels in femurs of sham and ovariectomized (OVX) rats fed casein, shark, or cod diets. BMD was measured by DXA, and the femur was divided from top to bottom into 20 slices. Data for slices 3 and 18 are shown. (A) Proximal metaphysis (slice 3). (B) Distal metaphysis (slice 18). Data were shown as means ± SD (n = 6 or 8). *Significant difference from OVX casein group at $P < 0.05$. †Significant difference from OVX casein group at $P < 0.01$.

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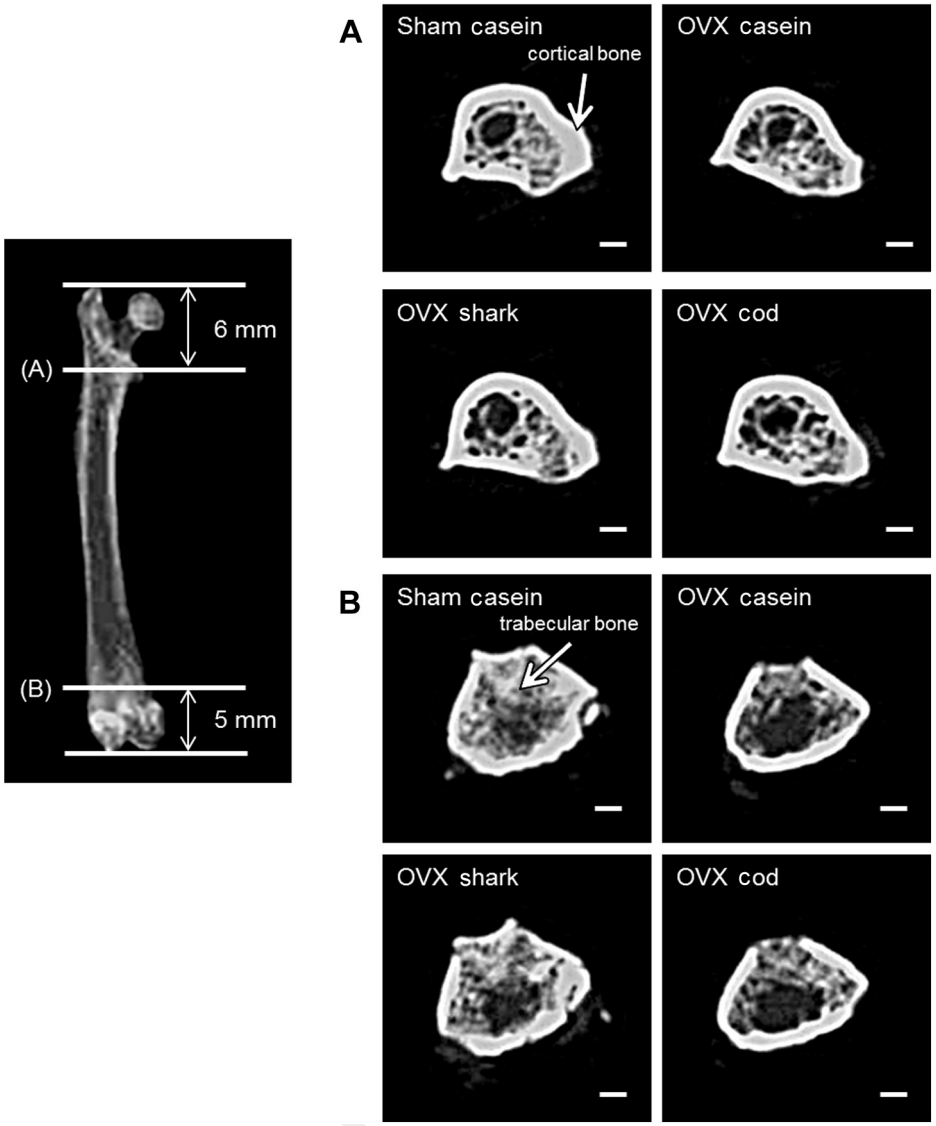

Fig. 3. Representative features of quantitative computed tomography images of cross sections of (A) proximal metaphysis and (B) distal metaphysis of femur. Femur specimens were analyzed using cross-sections taken 6 mm from the proximal end and 5 mm from the distal end. The arrow in (A) indicates cortical bone area. The arrow in (B) indicates trabecular bone area. Morphometric parameters of the proximal and distal metaphysis of femurs are described in Table 4. Bars 1 mm.

obviously different from casein because it contains lower levels of the amino acids glutamic acid/glutamine, proline, tyrosine, and valine, and higher levels of glycine, arginine, alanine, and methionine. Furthermore, shark protein was characterized as containing relatively high levels of isoleucine and leucine compared with cod protein.

Properties of SH and CH

Molecular-weight distributions of SH and CH were analyzed by gel filtration chromatography, as shown in Figure 1. The average molecular weights of SH and CH were 221 and 314, respectively.

Effect of shark protein on the basic parameters and BMD

The rat experimental data are shown in Table 3. Although the final weight and food intake increased significantly, the uterine weight decreased significantly in the OVX casein group compared with the sham casein group. However, there was no significant difference between OVX groups in final weight, food intake, uterine weight, femoral bone weight, and bone length.

To examine whether the effects of shark protein and cod protein differed between trabecular and cortical bone, the effects of these diets on BMD were compared in different slices along the femur. Figures 2A and 2B show the BMD values of slice numbers 3 and 18, respectively, from the experimental rat right femurs. The OVX shark group showed an increase in BMD at the epiphysis that was composed mainly of trabecular bone. The BMD of the OVX shark group was higher than that of the OVX casein group.

Representative pictures of QCT images of the proximal metaphysis and the distal metaphysis of the femur are shown in Figure 3. Decreases in cortical bone were seen in the proximal femur (Fig. 3A) of the OVX casein group compared with those of the sham casein group. The OVX shark group showed increases in cortical bone in the proximal femur similar to the sham casein group. Furthermore, decreases in trabecular bone were seen in

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Table 4
Quantitative computed tomography measurements of femurs in rats fed casein, shark, or cod diets*

	Sham	OVX		
	Casein (n = 6)	Casein (n = 8)	Shark (n = 6)	Cod (n = 8)
Whole				
Total BMD (mg/cm^3)	779.2 ± 22.8†	683.0 ± 34.4	708.0 ± 21.0	703.7 ± 13.6
Cortical BMD (mg/cm^3)	1111.8 ± 5.6†	1070.2 ± 24.8	1084.8 ± 13.8	1083.6 ± 13.5
Trabecular BMD (mg/cm^3)	489.4 ± 25.4†	402.5 ± 20.9	423.0 ± 15.9	416.2 ± 13.6
Proximal				
Total BMD (mg/cm^3)	843.4 ± 30.0†	745.9 ± 33.2	776.9 ± 25.4	753.1 ± 21.8
Cortical BMD (mg/cm^3)	1089.4 ± 7.9†	1038.0 ± 25.2	1964.9 ± 8.2‡	1050.1 ± 15.9
Trabecular BMD (mg/cm^3)	577.4 ± 31.7†	499.5 ± 25.8	511.6 ± 27.0	501.1. ± 24.3
Central				
Total BMD (mg/cm^3)	837.6 ± 37.4†	773.3 ± 38.2	798.9 ± 20.2	805.7 ± 20.7
Cortical BMD (mg/cm^3)	1259.8 ± 8.9‡	1235.9 ± 17.5	1246.0 ± 14.8	1246.7 ± 13.1
Trabecular BMD (mg/cm^3)	240.0 ± 15.5‡	223.1 ± 6.4	227.6 ± 11.1	227.6 ± 14.3
Distal				
Total BMD (mg/cm^3)	695.3 ± 21.3†	583.4 ± 37.6	602.7 ± 20.9	606.8 ± 12.7
Cortical BMD (mg/cm^3)	995.2 ± 9.8†	940.8 ± 33.1	942.4 ± 21.5	957.3 ± 15.0
Trabecular BMD (mg/cm^3)	531.0 ± 24.8†	414.4 ± 26.6	444.1 ± 14.4‡	134.3 ± 13.9

BMD, bone mineral density; OVX, ovariectomized rats; Sham, sham-operated rats
* Data are shown as means ± SD (n = 6 or 8).
† Significant difference from OVX casein group at $P < 0.01$.
‡ Significant difference from OVX casein group at $P < 0.05$.

the distal femur (Fig. 3B) of the OVX casein group compared with those of the sham casein group. The OVX shark group showed increases in trabecular bone in the distal femur to levels similar to the sham casein group.

Total BMD, cortical BMD, and trabecular BMD of the right femur were measured by QCT. Total BMD, cortical BMD, and trabecular BMD decreased significantly in the OVX casein group compared with the sham casein group (Table 4). The OVX shark group showed an increase in cortical BMD at the proximal sites of the femur. Furthermore, the OVX shark group showed an increase in trabecular BMD at the distal sites.

Effects of SH on 1,25(OH)2 D_3-induced osteoclast formation

Osteoclast differentiation was estimated by TRAP-positive multinucleated cell formation. Osteoclast formation was induced by 1,25(OH)2 D_3. As shown in Figure 4A, osteoclast formation was hardly detected when no 1,25(OH)2 D_3 was added. However, when 1,25(OH)2 D_3 was added, osteoclast formation was seen. With the addition of SH, osteoclast formation was inhibited. The number of differentiated osteoclasts induced by 1,25(OH)2 D_3 was 28.7 ± 4.8 cells per well. The addition of SH reduced the number of TRAP- positive multinucleated cells to 35% (Fig. 4B).

Effect of SH on RANKL-induced osteoclast formation

Although the bone marrow cultures were carried out in conditions that promoted osteoclastogenesis, it is still possible that the effects seen depended on the presence of other cell types. Therefore, we repeated these studies in RAW264 cells, a clonal cell line with a preosteoclast phenotype. Inhibition of osteoclast development was again observed with SH at 10 mg/mL with the same efficacy seen in the bone marrow cultures (Fig. 4C). This suggests that SH has a direct effect on preosteoclast cells and does not depend on the presence of other cell types. The number of TRAP-positive cells declined by 23% in SH-treated cultures. The number of TRAP-positive cells also declined by 15% in CH-treated cultures.

Discussion

Results of the amino acid analyses of shark protein and cod protein, showed no significant differences (Table 2) because they are both derived from white fish. Because the protein is largely muscle, it can be assumed that mainly actin and myosin were digested. However, differences in molecular weight were found between SH and CH when treated with Alcalase®2.4 L FG and umamizyme G. The sharks had been caught in tuna trawl nets, thus they had been stored on ice for a long time. In contrast, the cod had been caught during inshore fishing, so they had been frozen quickly after being caught. Several biochemical and enzymatic changes are triggered in fish muscle soon after death, so that the peptides lower in molecular weight [14]. Enzymes are commonly used as food-grade additives dissolved in water and these easy to handle materials will be useful as health foods in the future.

The ovariectomies were deemed successful as visually confirmed by the high degree of atrophy found in all corresponding ovaries. The greater body mass accretion of the castrated rats was in agreement with other reports [15,16] and is understood to result from the absence of estrogen, which regulates body fat deposition and leads to more efficient food conversion, the latter characteristic being verified despite the ovariectomized animals being pair-fed with sham rats [16].

Figure 2 shows the lower BMD of the epiphysis from the OVX casein group compared with the sham casein group. The pathology of the epiphysis from the OVX casein group showed a lower density of trabecular bone than the sham casein group (Fig. 3). Ovariectomy stops the secretion of estrogen and decreases BMD, especially in the trabecular bone and under the epiphyseal plate. The OVX casein group showed symptoms of osteoporosis. However, BMD levels in the OVX shark group were higher than in OVX casein group. Bone formation and bone resorption were most active in the trabecular bone area (epiphysis). As the amino acid content of the shark protein is practically identical to that of cod protein, the different responses observed in both the OVX shark and OVX cod groups could only be explained by the physiological functions likely to be linked to some of the peptides.

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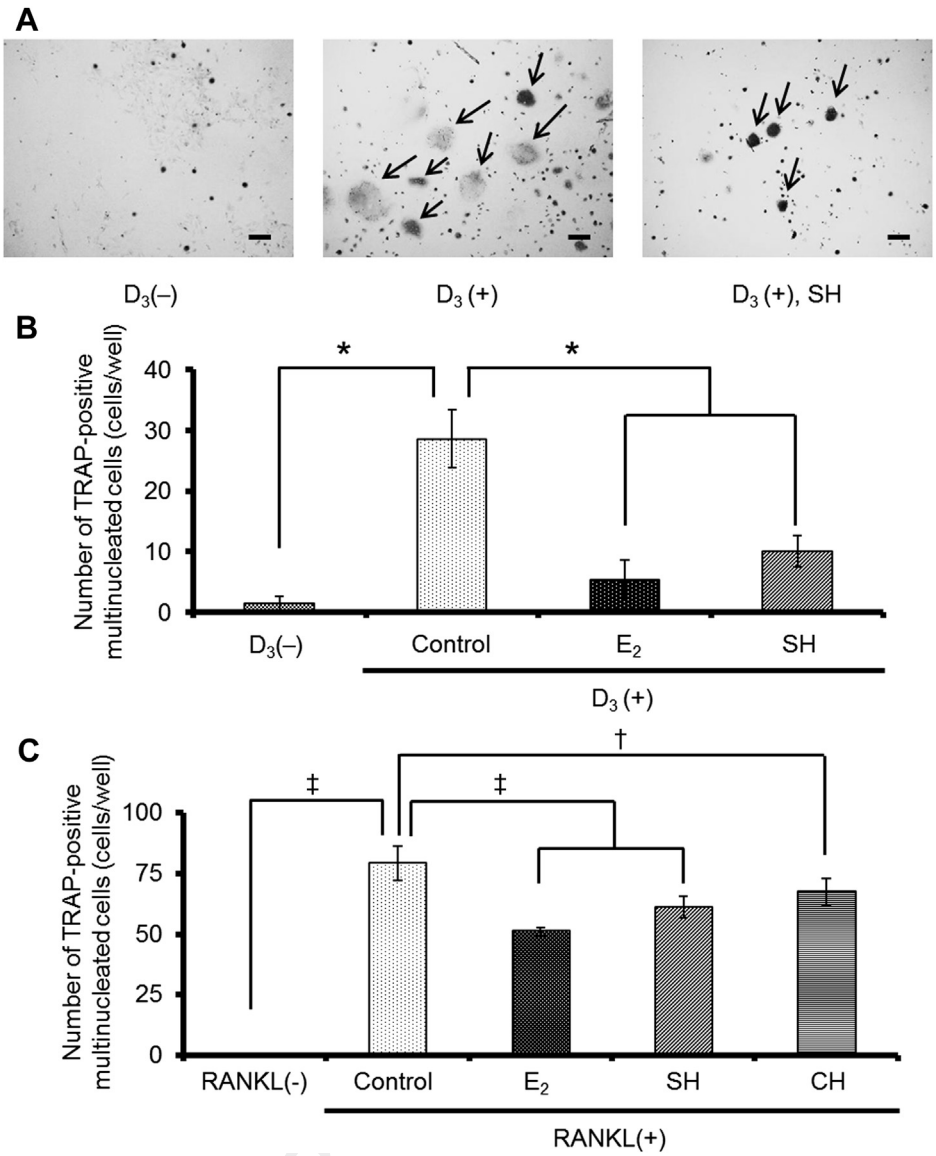

Fig. 4. Inhibition of osteoclast formation by shark protein hydrolysates (SH). (A) SH was added to bone marrow cells with 1 α,25-dihydroxyvitamin D_3 (D_3) in 48-well plates. After incubation for 6 d, osteoclasts (arrows) were stained using the tartrate-resistant acid phosphatase (TRAP) method. Bars 100 μm. (B) TRAP-positive multinucleated cells that had more than three nuclei were counted in bone marrow cells. (C) RANKL-stimulated TRAP-positive multinucleated cells in RAW 264 cells. Data were shown as means ± SD (n = 3). E2, 17 β-estradiol; CH, cod protein hydrolysates. *Significant difference from D_3(+) control at $P < 0.01$. †Significant difference from RANKL(+) control at $P < 0.05$. ‡Significant difference from RANKL(+) control at $P < 0.01$.

It is generally accepted that milk is an excellent source of calcium and contains several profitable components for calcium resorption in the intestine, such as lactose and phosphopeptides, formed by the proteolytic digestion of milk casein [17–19]. However earlier studies confirmed that calcium resorption was unaffected by shark protein (data not shown). The shark protein was digested into amino acids and peptides and absorbed in the body. This might have had a direct effect on osteoclast and osteoblast activity, thus increasing the BMD. Accordingly, we prepared low-molecular-weight peptides from shark and cod protein using Alcalase®2.4 L FG and umamizyme G for in vitro studies.

In bone remodeling, bone resorption by osteoclasts occurs before bone formation by osteoblasts. Estrogen deficiency accelerates bone resorption, and trabecular bone becomes porous [20]. Therefore, suppression of bone resorption leads to arrested trabecular bone loss under estrogen deficiency. In the presence of transforming growth factor-β and macrophage colony-stimulating factor, osteoclast progenitor cells differentiate from stem cells [21–24]. In the presence of macrophage colony-stimulating factor and RANKL, preosteoclasts differentiate from progenitor cells [25,26]. RANKL is a membrane-bound factor that is produced by osteoblasts and stromal cells in response to a variety of signals such as 1,25(OH)2 D_3 and parathyroid hormone. RANKL acts on osteoclast progenitors to induce osteoclast differentiation [27,28]. Although shark protein is thought to inhibit bone resorption in animals, its target cells in relation to bone resorption and its mode of action have not been fully elucidated. We determined the effects of SH on the function and differentiation of osteoclasts. In this study we demonstrated that SH inhibits TRAP-positive multinucleated cell formation induced by 1,25(OH)2 D_3 in cultures of bone marrow cells and

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RANKL in cultures of RAW264 cells. Our results suggest that the suppressive effect of SH on bone resorption might mainly result from its direct inhibitory effect on osteoclast differentiation (Fig. 4). We demonstrate the effect of SH on osteoclast differentiation and that its inhibitory effect on differentiation is partially responsible for its suppressive effect on bone resorption in cell culture. Similarly, in this study we demonstrated that CH inhibits TRAP-positive multinucleated cell formation induced by RANKL in cultures of RAW264 cells. However, we included an effective material such as shark protein, but the levels may have been very low because no effect was seen in vivo.

It has been reported that bioactive peptides derived from marine species improve BMD levels in marine collagen peptides [29], and leucine–lysine–proline [30]. It has also been reported that these peptides stimulate osteoblasts, thus increasing bone formation. However, we demonstrate here that SH acts directly on the differentiation of osteoclasts.

Conclusion

We show that ingesting shark protein increases BMD in osteoporosis, apparently through the inhibition of osteoclast formation. Therefore, we would like next to identify and isolate a peptide in shark protein inhibiting osteoclast differentiation.

Acknowledgments

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