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ORIGINAL ARTICLE

Fusako Maehira · Yoshirou Iinuma · Yukinori Eguchi
Ikuko Miyagi · Shoei Teruya

Effects of soluble silicon compound and deep-sea water on biochemical and mechanical properties of bone and the related gene expression in mice

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Abstract Silicon has been known as an essential element for bone formation. The silicon contents of sea water increase with increasing of depth: 1.8 ppm Si in deep-sea water (DW) at 612 m in depth versus 0.06 ppm in surface sea water (SW). The effects of soluble silicon (Si) and DW from which NaCl was eliminated were studied in comparison with tap water (TW) and SW in cell cultures and in animal experiments using the control strain of senescence accelerated mouse, SAMR1. Si at 10 ppm as sodium metasilicate or 10% DW in the α-MEM medium stimulated cellular viability, marker enzymes of osteoblast and osteoclast cell lines, and the $^{45}CaCl_2$ uptake in those cells in comparison with the medium control. After weanling SAMR1 were maintained for 6 months on a diet containing 200 ppm Si and 39% of DW and SW, DW and Si improved bone biochemical indices such as femoral weight, mineral and collagen content, and marker enzymes of bone formation and resorption as well as mechanical properties as compared to TW. In the femoral bone marrow of SAMR1, the mRNA expression of bone morphogenetic protein-2 (BMP-2), interleukin-11 (IL-11), and runt-related transcription factor 2 (Runx 2), which stimulate osteoblast development as well as type I procollagen (COL1A1) mRNA, were significantly increased in both DW and Si groups. The expressions of both osteoprotegerin (OPG) and receptor activator of NF-κB ligand (RANKL) were also elevated, resulting in distinct increases of the OPG/RANKL ratio in both DW and Si groups. The results indicated that a soluble silicate and deep-sea water as its natural material stimulated cell growth in both osteoblasts and osteoclasts in cell culture and promoted bone metabolic turnover in favor of bone formation through stimulation of the related mRNA expression in animal experiments.

Key words soluble silicon · deep-sea water · bone metabolic turnover · mRNA expression · mice

Introduction

Trace mineral involvement in bone metabolism, particularly Cu, Mn, and Zn, has been extensively studied in animals [1]. The essentiality of silicon (Si) is demonstrated in chicks and rats through effects on growth and skeletal development [2,3]: abnormalities involving articular cartilage and connective tissue are produced in chicks on silicon-deficient diets. The functional role of Si is indicated by its localization in active calcification sites in young bone [4] and also its highest distribution in femurs among tissues of rats and monkey [5], where silicon in the femur head containing the epiphysis is ten times as high as in the more mature shaft. Other studies have suggested Si–gonadal interaction. Ca-Mg imbalances or bone loss caused by hormonal imbalances, which are themselves caused by natural (aging) and experimental (ovariectomy) factors, are improved by administration of either organic or inorganic Si [6–8], implying the potential therapeutic application of Si in the treatment of involuntary osteoporosis. Administrated sodium metasilicate significantly increases the luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels in the hypohysis of female rats in diestrus and proestrus [6]. The molecular mechanism of the primary role of silicon in bone metabolism remains to be determined. Silicon in serum and tissues is present almost entirely as free soluble monosilicic acid, which is freely diffusible across the cellular membrane [4,9], resulting in its rapid renal clearance. An average 50% of the ingested dose was absorbed and eliminated with half-lives of 2.7 h and 11.3 h for 90%

F. Maehira (✉) · Y. Iinuma · I. Miyagi
Laboratory of Biometabolic Chemistry, School of Health Sciences, Faculty of Medicine, University of the Ryukyus, 207 Uehara, Nishihara, Okinawa 903-0215, Japan
Tel./Fax +81-98-895-1277
e-mail: fmaehira@med.u-ryukyu.ac.jp

Y. Eguchi
Research Laboratory Center, Faculty of Medicine, University of the Ryukyus, Nishihara, Okinawa, Japan

S. Teruya
Okinawa Industrial Technology Center, Gushikawa, Okinawa, Japan

and 10%, respectively [10,11]. The latter slower component may represent an intracellular fraction that may be involved in its physiological function, and the element was found to be equally distributed among subcellular fractions [5]. Such rapid clearance of inorganic silicon may be considered a potential benefit in the long-term treatment of osteoporotic disorders without excessive retention and accumulation in the body. Concentrations of nutrient constituents of sea water such as nitrogen, phosphorus, and silicon increase with increasing depth [12,13]; 64 μM SiO_2 (1.8 ppm Si) in deep-sea water (DW) from 612 m in depth versus 2.1 μM (0.06 ppm) in surface sea water (SW) by our analysis. In this study, the effects of soluble silicon (Si) and DW as its natural material were examined in comparison with tap water (TW) and SW, in cell culture and in animal experiments using the control strain of senescence accelerated mouse, SAMR1. In a recent in vitro cell culture study, the effects of orthosilicic acid on the expression of osteoblastic-related genes have been reported using semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) [14]. The animal study for bone metabolism in this study included biochemical and mechanical properties of bone and related gene expressions in mouse femurs: the expression of osteoblastogenesis- and osteoclastogenesis-specific gene markers using quantitative real-time PCR.

Materials and methods

Preparation of desalinated sea waters

DW from 612-m depth and SW from 15-m depth were desalinated with an electrodialytic desalination units (Acilyzer G3; Asahi, Tokyo, Japan) equipped with monovalent cation-exchange membrane, desalinating monovalent cations up to an electro-conductance of 10 mS/cm. Sea water in this study, therefore, indicates desalinated sea water. Before and after desalination of DW and SW, Na, Ca, Mg, and Si levels were analyzed: sodium by flame spectrophotometry, calcium and magnesium by atomic absorption spectrophotometry, and soluble silicate by the molybdate method, respectively (Table 1). Bioavailable soluble silicate, monosilicic acid, reacts with molybdate reagent to give a yellow color, whereas amorphous silica or polysilicic acid do not react with this reagent [15]. An available soluble silicon compound, sodium metasilicate $Na_2SiO_3 \cdot 9H_2O$, was used in the cell culture and animal studies.

Cell cultures

A murine preosteoblastic cell line, MC3T3-E1, bone marrow-derived stromal cell line, ST2, and bone marrow-derived preosteoclastic cell line, MLC-7, were purchased from RIKEN (Tsukuba, Japan). MC3T3-E1 cells and ST2 cells were maintained in α-minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, and 100 μg/ml streptomycin, at 37°C in 5% CO_2/95% air. MC3T3-E1 cells at 3 days subculture, which were maintained in α-MEM supplemented with 10% FCS, were made “quiescent” by another 3-day or 1-day incubation in fresh α-MEM for osteoclast-like cells and McCoy’s 5A medium for MLC-7 cells with 0.4% FCS before various analyses.

Bone marrow (BM) cells were obtained from femora and tibia of 12-week-old male SAMR1 mice by flushing the marrow cavity with α-MEM, and erythrocytes in bone marrow cells were hemolyzed in 17 mM Tris-HCl buffer, pH 7.5, containing 0.8% NH_4Cl, followed by rinsing twice with phosphate-buffered saline (PBS) [16]. BM cells (1.5×10^6) were cocultured with bone marrow-derived stromal cell line ST2 (4×10^4) in α-MEM containing 10% FCS, 10 nM 1,25-dihydoxyvitamin D_3 (1,25 $(OH)_2$ D_3), and 100 nM dexamethasone in a plastic dish (34 mm × 14 mm) at 37°C to achieve osteoclast-like cell differentiation [17]. Cultures were fed every 3 days by replacing 0.4 ml old medium with fresh medium. After culture of 8–10 days, cells were fixed in 10% formalin neutral buffer solution for 10 min and stained for tartrate-resistant acid phosphatase (TRACP) [17,18] to confirm the formation of osteoclast-like cells. A preosteoclastic MLC-7 cell line, expressing fully differentiated osteoclast markers, such as calcitonin receptors, vitronectin receptors, TRACP, and vacuolar H^+-ATPase without losing undifferentiated osteoclast precursor cell phenotypes [19], was maintained in McCoy’s 5A medium supplemented with 20% FCS. As osteoclast properties of MCL-7 cells were further stimulated when they were cocultured with ST2 cells [19], MCL-7 cells (2×10^5) were cocultured with ST2 (4×10^4) in McCoy’s 5A containing 10% FCS, 10 nM 1,25-dihydoxyvitamin D_3 (1,25 $(OH)_2$ D_3), and 100 nM dexamethasone in a well of 24-well plates. In the preliminary study, the effects of DW or Si on the cell viability or the activities of alkaline phosphatase activity (ALP) for osteoblasts and TRACP for osteoclasts were then examined by culturing cells in α-MEM or McCoy’s 5A containing 0.4% FCS and various concentrations of DW (0%, 2.5%, 5%, 10%, 20%) or Si (0, 1, 5, 10, 25 ppm) for another 4 days. Cell viability was analyzed in triplicate culture wells by

Table 1. Minerals in sea water before and after treatment by electrodialysis with monocationic membrane

Minerals	Na (mEq/l)		Ca (ppm)		Mg (ppm)		Si (ppm)	
	DW	SW	DW	SW	DW	SW	DW	SW
Before	458	436	388	388	1300	1360	1.78	0.06
After	16	13	232	232	1060	1060	1.72	—
%Removal	96.5	97.0	40.2	40.2	18.5	22.1	3.4	—

DW, deep-sea water; SW, surface sea water

using soluble tetrazolium dye WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-terazolium sodium salt; DOJINDO, Kumamoto, Japan) with absorbance at 450 nm according to the protocol provided by the manufacturer. For analyses of marker enzymes, cultures in triplicate wells were rinsed twice with 0.9% saline, then 80 μl assay buffer at pH 10.5 for ALP and at pH 6.0 for TRACP together with 20 μl 5% NP-40 were added and sonicated twice for 10 s on ice. Following centrifugation at 10 000 rpm for 10 min at 4°C, the soluble fraction was assayed in duplicate per culture well for ALP activity at pH 10.5 for 15 min, and TRACP activity at pH 4.9 for 60 min with absorbance at 500 nm by the Kind–King method. The cell viability or the activities of marker enzymes were expressed as mean optical density (OD)/well ± SD of triplicate culture wells.

Calcium uptake in osteoblasts and osteoclast-like cells was also examined in triplicate culture dishes using $^{45}CaCl_2$ (5–50 mCi/mg calcium; Amersham Biosciences, UK) [20]. In conjunction with the animal experiment, we used osteoclast-like cells derived from bone marrow of SAMR1 rather than the established MLC-7 cell line as osteoclasts. After incubating the cells with 0.88 μCi ^{45}Ca in medium per dish for 3 h at 37°C in an incubator, the dishes were placed on ice and the medium was removed. Cells were washed rapidly three times with cold Krebs–Ringer, and then they were solubilized in 200 μl 0.05% Triton-X 100 solution. After centrifugation at 10 000 rpm for 5 min, the radioactivity of 100 μl supernatant was measured by liquid scintillation counter and ^{45}Ca uptake was expressed as μg Ca/mg cellular protein. Cellular protein was analyzed at duplicates by Protein Assay kit (Bio-Rad, Hercules, CA, USA).

Animal experiments

A murine model of senile osteoporosis, SAMP6, and its control, SAMR1, were provided by the Council for SAM Research, Kyoto University, where these mouse strains were developed. The experimental protocols of this study were approved by our Institutional Animal Studies Committee. Mice were maintained for 6 months on a semisolid diet (Rodent Diet CE-2 powder; CLEA Japan, Tokyo) ad libitum containing 39% of either TW or DW, SW from which NaCl was eliminated, and the diet supplemented 200 ppm Si to the TW diet, which had an Si dose in the range of 100–500 ppm, as used in previous studies without toxic actions [2–4,8]. In general opinion and in our experience with animal experiments, mice have a preference for a lobe-like softness of semisolid diet containing 35%–40% moisture depending on the nature of the powder diet, and 39% moisture was used in this study. At the end of the experiments, urine was collected over a 24 h period with our handmade urine traps and used to measure the urinary content of hydroxyproline (OHPro) [21]. Urinary OHPro was corrected for creatinine excreted in urine. Supernatants from homogenates of the right tibia, after removal of its marrow, at pH 10.5 for ALP and at pH 6.0 for TRACP were used to measure enzyme activities by the procedures described in the cell culture, and the activities were expressed as KA-U per mg protein. Mechanical tests were performed on the left femur, after cleaning of soft tissue, in three-point bending using an EZ Test-100N (Shimazu, Kyoto, Japan) [22]. After mechanical tests, the wet, dry (100°C, 24 h), and ash (500°–550°C, 24 h) weights of the left femur were measured, and calcium (Ca) by the o-CPC method and phosphorus (P) by the phosphomolybdenum blue method [23] followed after dissolving ash in acid, respectively. Acid digests of the right femur after removal of its marrow were used to measure OHPro. Collagen content was determined as OHPro by oxidizing OHPro into pyrrole followed by coupling with *p*-dimethyl-aminobenzaldehyde [24].

At the start of the present study, male SAMR1 or SAMP6 1 month old were divided into seven or three groups of seven each. From seven groups of SAMR1, three groups (TW, DW, and SW) were fed for 3 months until 4 months old with their peak bone mass, and the remaining four groups (TW, DW, SW, and Si) were fed for a further 3 months until their age was 7 months old with a gradual decrease of bone mass after reaching the peak. Accordingly, the study for the 6-month feeding experiment was performed on four groups of SAMR1 and three groups (TW, DW, and SW) of SAMP6. The SAMR1 at 4 months old did not show statistically significant changes among groups except increases in the femoral ash weight of DW and SW groups, probably because the growing mice were at their peak bone mass. In addition, there were slight changes without statistical significance observed in the results of biochemical and mechanical properties of SAMP6, in which low peak bone mass is genetically controlled. Consequently, the results for SAMR1 fed for 3 months and SAMP6 were omitted from the present study. Instead, this report focused on the effects of soluble Si and DW on the natural age-associated changes in the incipient stage of senescence at 3 months after reaching the peak bone mass using SAMR1 with age of 7 months, as the main criterion used for the pedigree selection is the degree of senescence in the mice at 8 months of age [25].

RNA isolation and gene expression analysis

Total RNA was isolated from bone marrow of the right femora and tibiae at the end of animal experiments using a NucleoSpin RNA II kit (Macherey-Nagel, Dren, Germany). Two micrograms of RNA was used for cDNA synthesis by reverse transcription reaction with an Omniscript RT kit (Qiagen, Germantown, MD, USA). A quantitative real-time (RT)-PCR was performed using the Mx3000P real-time PCR system (Stratagene, La Jolla, CA, USA) and fluorescent dye SYBR Green (Brilliant SYBR Green QPCR Master Mix; Stratagene) to detect a double-strand DNA amplicon. Gene expression was analyzed using the following pairs of primers: BMP-2, (forward) 5′-CTTC TAGTGTTGCTGCTTCC-3′ and (reverse) 5′-CTCAACT CAAATTCGCTGAG-3′; IL-11, (forward) 5′-CTCTT GATGTCTCGCCTG-3′ and (reverse) 5′-CTAGGATG

GCATGAGCTG-3′; Runx2/Cbfa 1, (forward) 5′-GCTTC ATTCGCCTCACAAACA-3′ and (reverse) 5′-TGCAGTC TTCCTGGAGAAAGTT-3′; COL1A1, (forward) 5′-GAG ATGATGGGGAAGCTG-3′ and (reverse) 5′-ACCATC CAAACCACTGAAG-3′; OPG, (forward) 5′-GCACCTAC CTAAAACAGCAC-3′ and (reverse) 5′-GCTGCAAT ACACACACTCAT-3′; RANKL, (forward) 5′-GCTCTGT TCCTGTACTTTCG-3′ and (reverse) 5′-CTGCGTTTTC ATGGAGTC-3′; GAPDH, (forward) 5′-AACGACCCC TTCATTGAC-3′ and (reverse) 5′-TCCACCACATACT CAGCAC-3′. All RT-PCR reactions contained first-strand cDNA corresponding to 1–10 ng RNA. The PCR protocol included the following cycling conditions: 95°C denaturation for 10 min, then 45 cycles of 95°C denaturation for 30 s followed by 60°C annealing for 1 min, and 72°C extension for 30 s. Detection of the fluorescent amplicon was mostly done at the end of the 60°C annealing period. The sizes of PCR products for mouse Runx2, BMP-2, IL-11, COL1A1, OPG, RANKL, and GAPDH were 68, 143, 112, 137, 113, 100, and 191 bp, respectively. PCR products were subjected to a melting curve analysis and quantified with Mx3000*P* Software v. 1.20c (Stratagene). PCR results were normalized to the expression of GAPDH in the same samples. Triplicate analyses were performed for each sample.

Statistical analysis

Experimental results are reported as mean ± SD of triplicate independent samples for the cell culture study and of duplicate analyses for the animal specimens, unless otherwise indicated. Statistical analyses were made by comparing mean values by using either one-way analysis of variance (ANOVA) with SAS program or two-tailed *t* test as appropriate. Individual differences between groups were assessed using Duncan's multiple range test, in which the means with different letter are significantly different at $P < 0.05$.

Results

Effects on cellular viability, marker enzymes, and ^{45}Ca uptake in osteoblasts, osteoclasts, and osteoclast-like cells

In the preliminary study of examining the effects of DW and Si on both osteoblasts and osteoclasts, 10% additions of DW or 10 ppm Si as sodium metasilicate to α-MEM containing 0.4% FCS exhibited the best cellular growth and stimulatory effects on bone metabolic turnover with the highest activities of ALP in MC3T3-E1 osteoblasts and TRACP in MLC-7 osteoclasts and in osteoclast-like cells compared with the medium control (Fig. 1). The additions of 20% DW or 25 ppm Si to the media showed low viabilities and ALP activities in osteoblastic cell line. It is not clear in the present study whether the observed unfavorable results came from a toxic effect of Si or the disturbances of pH or salt balance in the medium. In the studies of the cell culture of chondrocytes and the tissue culture of chick embryos [4], the additions of Si to the media at concentrations up to 2.0 mM (56 ppm) gave stimulatory effects on prolyl hydroxylase activity, a measure of the rate of collagen biosynthesis.

The $^{45}CaCl_2$ uptake in MC3T3-E1 cells was enhanced by media containing 10 ppm Si (117%), 10% DW (126%), 10% DW plus 5 ppm Si (152%), and 10% DW plus 10 ppm Si (156%) as compared with the medium control (Fig. 2A). ^{45}Ca uptake in osteoclast-like cells was also stimulated by media containing 10 ppm Si (132%), 10% DW (166%), and

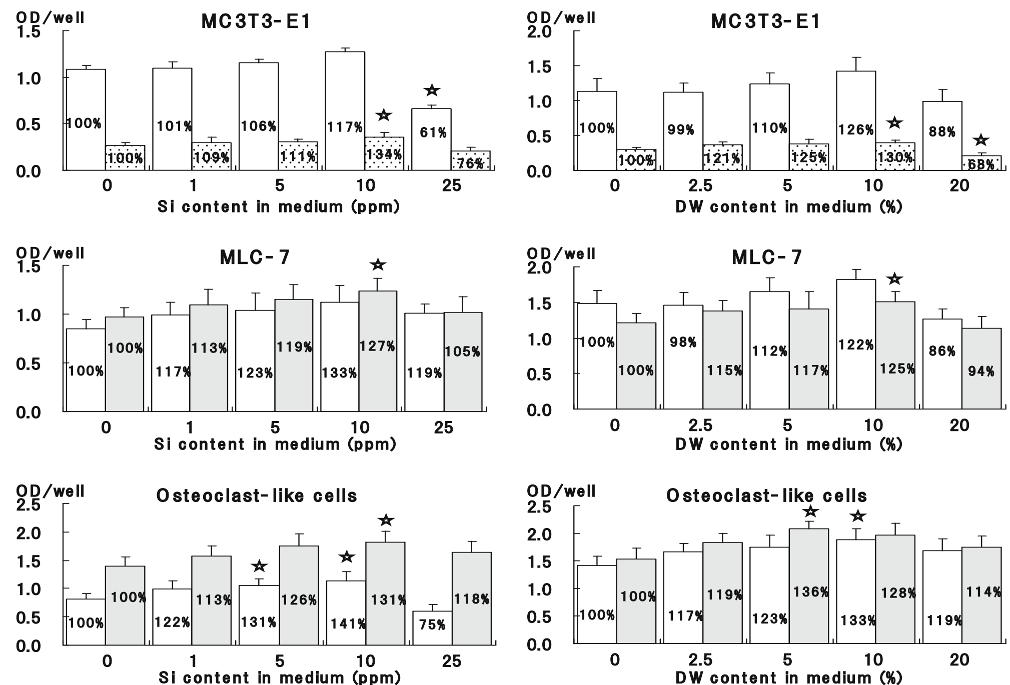

Fig. 1. Effects of soluble silicon and deep-sea water (DW) on cellular viability and marker enzymes of osteoblast (MC3T3-E1), osteoclast (MLC-7), and osteoclast-like cells. Bars are expressed as the mean optical density (OD)/well ± SD of triplicate culture wells. Absorbance at 450 nm for cell viability (*open bars*) and 500 nm for marker enzymes of alkaline phosphatase (ALP) (*stippled bars*) and tartrate acid-resistant phosphatase (TRACP) (*gray bars*) were used. *Open stars*, $P < 0.05$ vs. the medium control

10% DW plus 10 ppm Si (139%) as compared with each control (Fig. 2B). As increases in calcium concentrations in culture medium induced osteoclast formation [26], the Ca (+) control with 2.7 mM Ca against the medium control with 1.8 mM Ca was set for Ca increases derived from DW in the medium.

Effects on biochemical and mechanical properties of bone

All animals appeared healthy during the 6 months of the study period, and there was no significant difference in body weight among groups at the end of the experiment. The average daily uptake of minerals per mouse through the 6 months of the experimental period is shown in Table 2. Mg-rich sea water allowed both DW and SW groups 13% to 18% higher Mg uptake than the TW control group. Si uptake of 1.7 fold and 2 fold was observed in DW and Si groups as compared with that of the control, respectively. Only the DW group showed significant increases of 111% in femoral ash weight of SAMR1 compared with the control TW groups (Fig. 3A). In all test groups, femoral Ca and P contents increased significantly, from 7% to 12% and 6% to 9%, respectively (Fig. 3B). The activities of tibial ALP, a marker of bone formation, increased significantly, 14% and 20% in DW and Si groups, respectively, indicating stimulatory effects on bone formation by both DW and Si, respectively (Fig. 3C). On the other hand, prominent 32% suppression of TRACP activity, a marker of bone resorption, was observed in DW group (Fig. 3C). The femoral collagen content determined as OHPro increased significantly by 13% in the DW group (Fig. 4A), whereas its urinary excretion was suppressed to 17% (Fig. 4B), indicating the suppression of bone resorption.

Compared to TW control, three-point bending tests indicated that DW increased all four indices of mechanical properties—the strength of bone, structural stiffness, % deflection, and the amount of energy absorbed before breaking in femurs of SAMR1 (Fig. 5). Soluble Si also showed amelioration in the stiffness and the amount of energy absorbed.

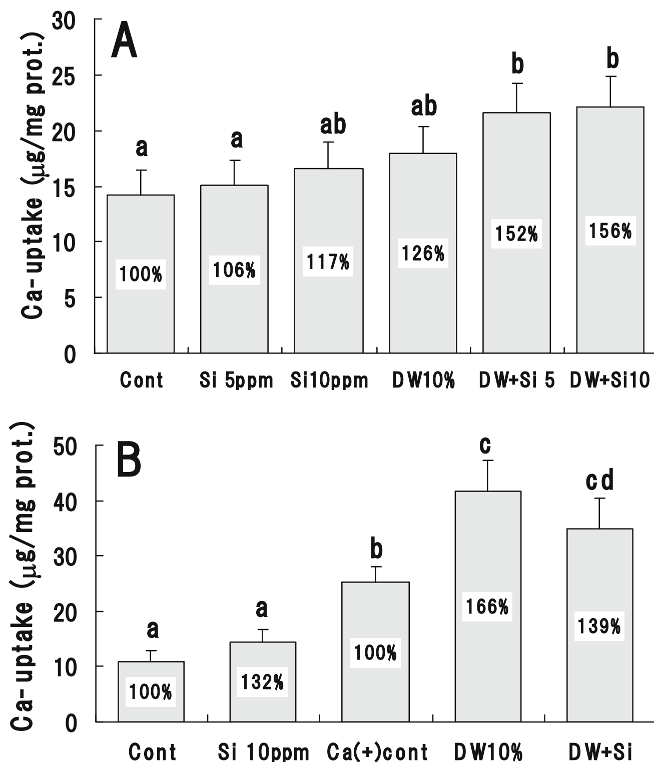

Fig. 2. Effects on ^{45}Ca uptake by osteoblasts (**A**) and osteoclast-like cells (**B**). Data are shown as the mean ± SD of triplicate culture wells. Because increases in calcium concentrations in culture medium induced osteoclast formation [24], Ca (+) control was set for Ca increases derived from DW in the medium as described in Methods. Means with different letter are significantly different at $P < 0.05$ (Duncan's new multiple range test)

Effects on gene expression related to osteoblastogenesis and osteoclastogenesis

Changes in the expression of bone formation- and resorption-specific markers are shown in Fig. 6. The mRNA expressions of osteoblastogenetic marker genes (A to D) were significantly increased 228% and 240% in DW and Si groups for BMP-2, 490% and 308% for IL-11, 385% and 242% for *Runx2*, and 491% and 545% for *COL1A1*, compared with the TW control group, respectively. For mRNA expressions related to osteoclastogenesis (E), OPG was increased 391% and 428% in DW and Si groups as compared to the control, respectively, while RANKL increased 182% and 122% in the two groups. The latter 122% increase of RANKL observed in the Si group was not statistically significant. When the ratio of OPG over RANKL was taken, 2.2-fold- and 3.5-fold-greater expression of OPG than RANKL was observed in DW and Si groups as compared with the control, respectively.

Table 2. Average daily uptake of minerals per mouse

Groups	TW		DW		SW		Si	
	g	(%)	g	(%)	g	(%)	g	(%)
Ca	0.066	(100)	0.064	(97)	0.067	(102)	0.066	(100)
Mg	0.020	(100)	0.022	(113)	0.023	(118)	0.020	(100)
Na	0.026	(100)	0.027	(102)	0.028	(106)	0.026	(100)
P	0.059	(100)	0.062	(106)	0.060	(101)	0.059	(100)
Si	9.5 μg	(100)	15.7 μg	(165)	9.9 μg	(104)	20.0 μg	(210)

TW, tap water; DW, deep-sea water; SW, surface sea water; Si, silicon

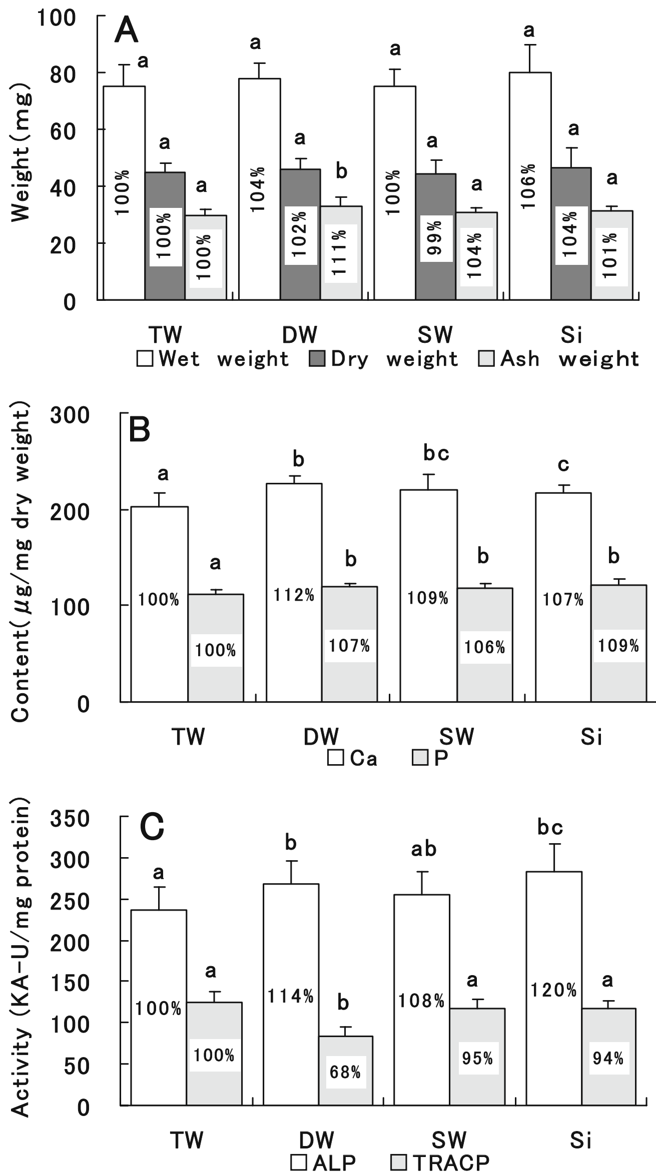

Fig. 3. Effects on femoral weights (**A**), Ca and P content (**B**), and tibial alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRACP) activities (**C**). Values shown are the mean ± SD for 7 animals in each group. Means with different letter are significantly different at $P < 0.05$. *DW*, deep-sea water; *SW*, surface sea water; *TW*, tap water; *Si*, silicon

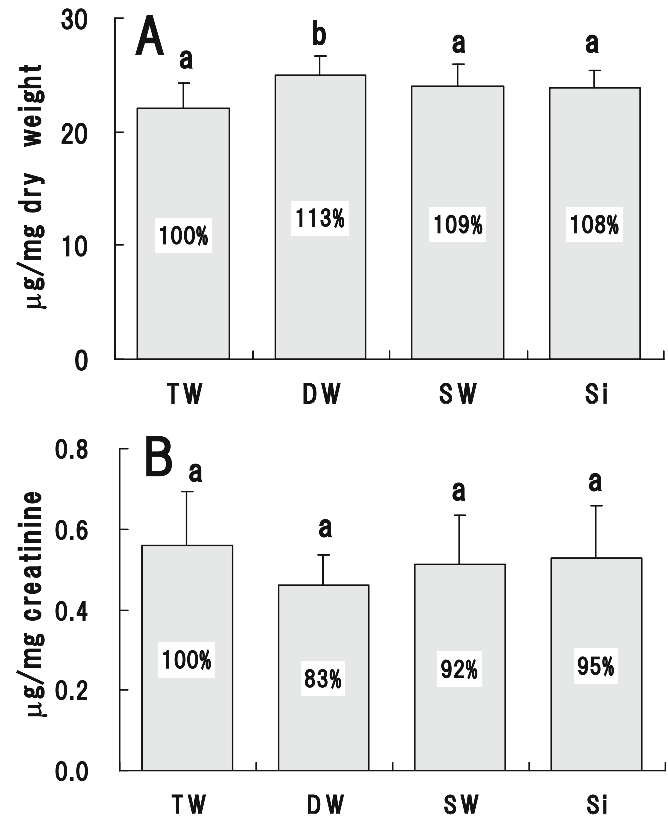

Fig. 4. Effects on femoral hydroxyproline content (**A**) and its urinary excretion (**B**). Values are the mean ± SD. Means with different letter are significantly different at $P < 0.05$

Discussion

The quality and quantity of bone are maintained by a continuous and coordinated process of remodeling that involves bone resorption by osteoclasts and subsequent bone formation by osteoblasts. Bone mass depends on the balance between resorption and formation within a remodeling unit. The present study indicated that the additions of either soluble Si or deep-sea water as its natural material to the culture medium enhanced cell viability, the activities of marker enzymes for differentiation of both osteoblasts and osteoclasts, and $^{45}CaCl_2$ uptake in both MC3T3-E1 cells and osteoclast-like cells. The enhancement of Ca uptake is beneficial to bone formation in osteoblasts [26,27] and activates a transcription factor for terminal differentiation of osteoclasts [26,28], indicating that Si and DW stimulate both osteoblastogenesis and osteoclastogenesis, i.e., bone turnover. The mechanism of the enhancement of cellular Ca uptake by Si is unclear. Silicon may act indirectly through the interaction with other metals. As monomeric silicic acid is a very weak acid with a pK_1 of 9.8, at physiological pH there can be no interactions with Ca^{2+} or Mg^{2+}, but interaction with Al^{3+} is possible [29,30]. Aluminum has the ability to displace Mg^{2+} or Fe^{3+} even in the presence of their high concentrations from key sites at which those metals are catalytic in various biological systems, and the role of silicic acid is to counteract deleterious effects of aluminum on enzymatic and Mg^{2+}-dependent processes. As aluminum will also bind to the phosphate group, there are possible effects of aluminum on the inositol phosphate and hence on intracellular Ca^{2+} homeostasis [29]. The mechanisms of the effect on the stimulation of cellular Ca uptake by soluble Si need to be elucidated.

In the animal study, deep-sea water from which NaCl was eliminated improved the biochemical properties of bone: an increase of bone ALP activity with a significant suppression of TRACP activity and increases of Ca and P content were observed, suggesting the stimulation of bone mineralization, which was reflected in an increase of ash weight, and also an increase of the bone collagen content

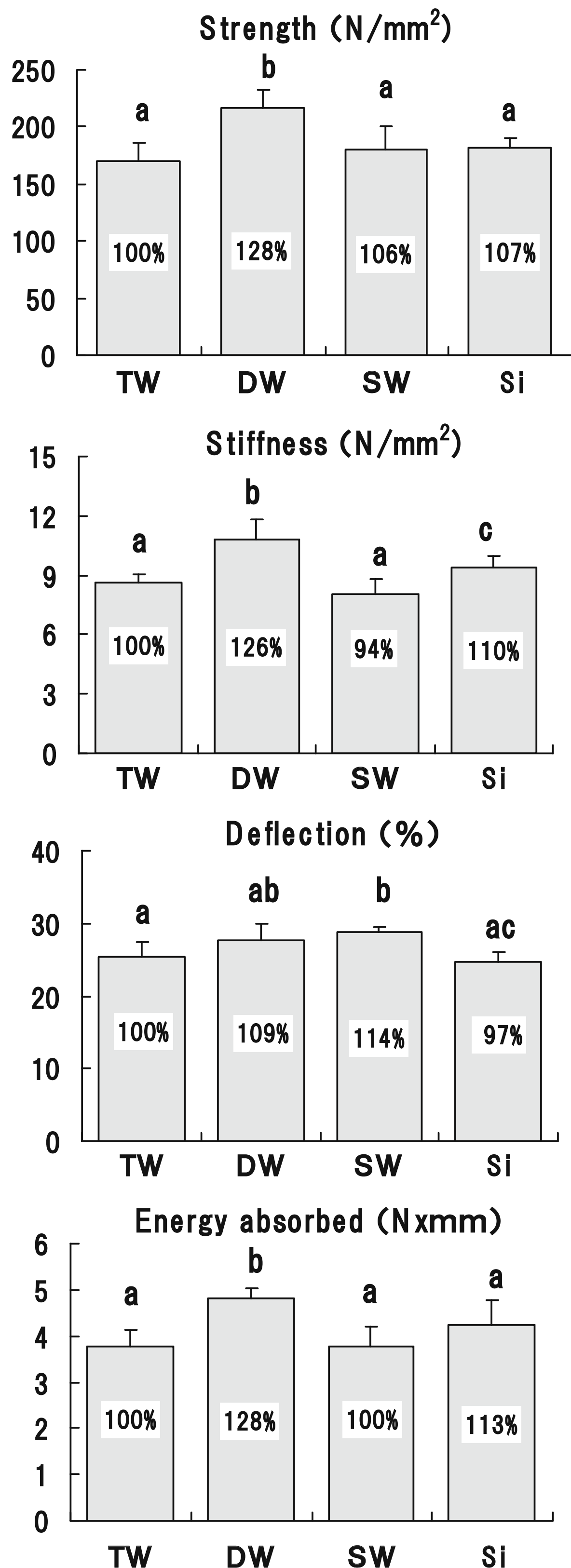

Fig. 5. Effects on mechanical properties of the femurs. Values are the mean ± SD. Means with different letter are significantly different at $P < 0.05$

with the suppression of urinary excretion of its decomposed products. Those changes were also observed in soluble Si to a lesser extent. The results obtained in the animal study reflected the stimulatory effects of DW and Si on both osteoblastogenesis and osteoclastogenesis, i.e., bone turnover, observed in the cell culture as the enhancement of cell viability, marker enzymes for bone formation and resorption, and Ca uptake in both osteoblasts and osteoclasts. Deep-sea water also exhibited improved biomechanical properties of bone; increased stiffness and strength of bone, and the amount of energy absorbed before breaking in femurs compared with the tap water controls. Si next to DW exhibited amelioration in biochemical and mechanical indices of bone. The observed difference in the effects on bone between DW and Si is probably caused by the content of other minerals important in bone metabolism such as Cu, Zn, and Mn [1] besides Si in DW.

In addition to the biochemical and biomechanical changes observed, the mRNA expressions of related genes were studied. Osteoblasts arise from common mesenchymal progenitors with chondrocytes, adipocytes, and myocytes, and differentiation to osteoblasts as specific phenotypes is regulated by various local factors among which BMP-2 is a potent osteogenic agent [31]. One of the downstream targets of BMP-2 signaling is Runx2/Cbfa 1 [32], a runt family transcription factor, the expression of which is activated by BMP-2 [32–34]. Runx2/Cbfa 1 regulates the expression of other genes that are activated during osteoblast differentiation, including osteocalcin [35], alkaline phosphatase [36], α1 and α2(I) collagen [37], and osteopontin [38]. These genes contain Cbfa 1-binding sites in their promoters, and Cbfa 1 is a master transcription factor for osteoblast differentiation, which concept is supported by the absence of bone formation in mouse embryos with the inactivated *Cbfa 1* gene [39]. Since among three isoforms of the *Cbfa 1* gene a third *Cbfa 1* isoform N-terminus beginning with the amino acid sequence MLHSPH has been detected in bone and osteoblast, but in no other tissues [38,40], we used the forward primer designed to the unique N-terminus to amplify Cbfa 1. IL-11 is a multifunctional cytokine [41] whose role has been originally described as the hematopoietic stimulation [42] and inhibition of adipogenesis [43] in bone marrow. Its role in osteoclast development was originally described mostly in vitro systems [44]. In a later in vivo study, intravenous administration of IL-11 in intact animals failed to increase bone resorption markers [45]. Transgenic mice overexpressing human IL-11 showed the stimulation of bone formation, resulting in increased bone mass and protected from aging-associated bone loss [46]. IL-11 alone and in combination with BMP-2 synergistically induced the osteoblastic differentiation of mouse mesenchymal progenitor cells [47], bone marrow stromal cell line and bone marrow cells [46]. In this study, mRNA expressions of those osteoblastogenetic marker genes, *BMP-2, IL-11, Runx2*, and *COL1A1*, were significantly increased in both DW and Si groups with lesser increases in the latter group as compared with the TW control group (Fig. 6A–D). Such difference in the gene expression observed between DW and Si groups is probably derived from mineral constituents in

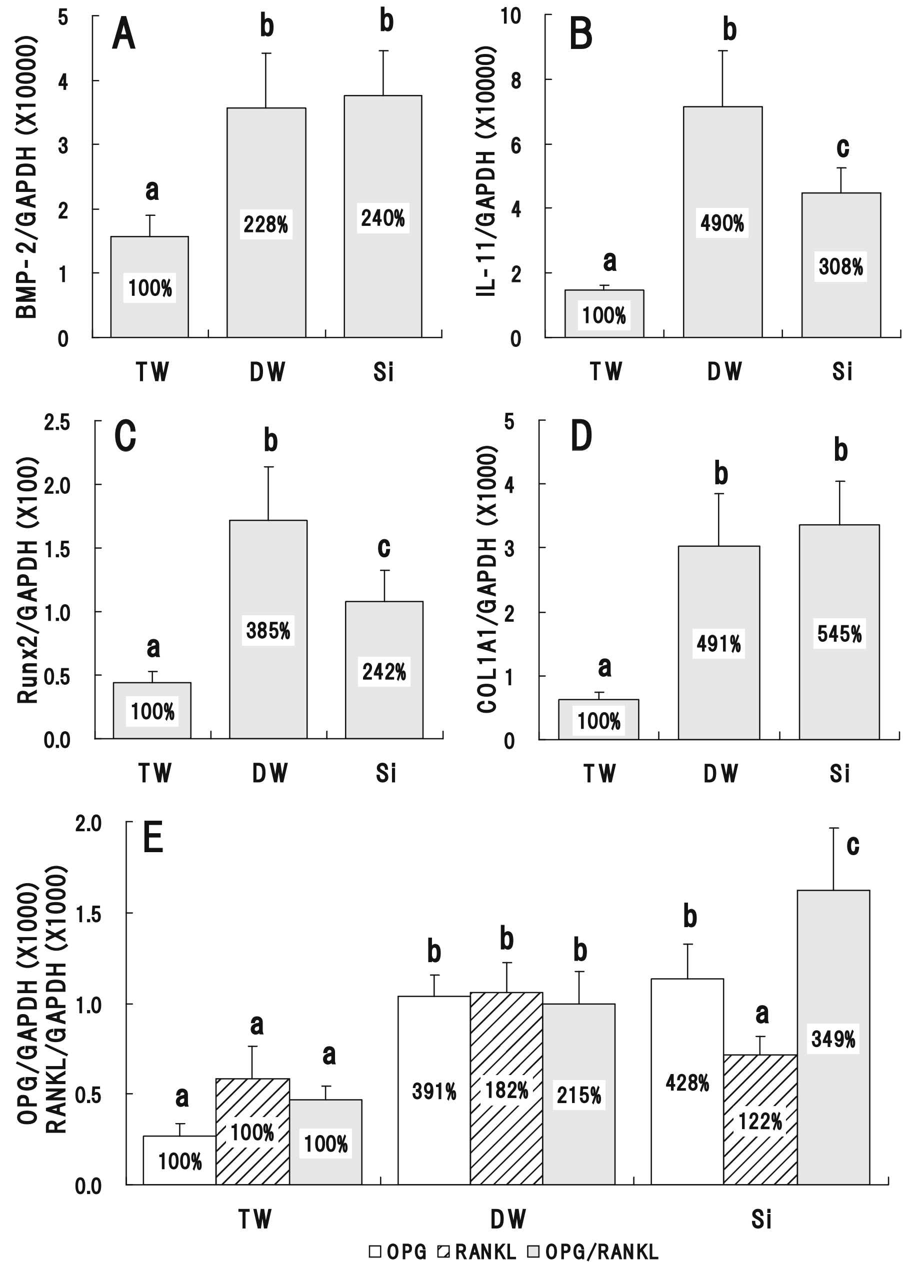

Fig. 6. Effects on the expression of mRNA related to osteoblastogenesis [**A**, bone morphogenetic protein (BMP)-2; **B**, interleukin (IL)-11; **C**, runt-related transcription factor 2 (Runx 2), **D**, type I procollagen (COL1A1)] and osteoclastogenesis (**E**, osteoprotegerin (OPG) for *open bars*, receptor activator of NF-κB ligand (RANKL) for *striped bars*, and OPG/RANKL ratio for *gray bars*). Values are the mean ± SD. Means with different letter are significantly different at $P < 0.05$

DW as described by the biochemical and mechanical properties. IL-11 stimulated transcription of the target gene, *Runx2*, for BMP-2, leading to enhanced osteoblastic differentiation in combination with BMP-2 and resulting in the increased mRNA expression of collagen.

Osteoclasts, multinucleated cells that resorb bone, are differentiated from hematopoietic progenitor cells. Osteoblasts/stromal cells play an essential role in osteoclastogenesis at bone-resorbing sites under the control of osteotropic hormones and local factors produced in the microenvironment by expressing on their cell surface a specific receptor activator of NF-κB ligand (RANKL)/ODF/OPGL/TRANCE (osteoclast differentiation factor/osteoprotegerin ligand/tumor necrosis factor-related activation-induced cytokine) [48]. Osteoblasts/stromal cells also produce osteoprotegerin (OPG)/osteoclastogenesis inhibitory factor (OCIF), a soluble decoy receptor for RANKL, which inhibits both the differentiation and activation of osteoclasts [49,50]. Regulation of the gene expression of those two factors, RANKL and OPG, produced by osteoblasts at the microenvironment of bone, is important for the osteoclast development, and various osteotropic agents are involved in the regulatory mechanisms [51]. In the present study, the balance between the levels of the OPG mRNA and RANKL mRNA in the bone marrow of mice was altered by feeding mice diets containing either DW or soluble Si (Fig. 6E) compared with the control diet: the expressions of both OPG and RANKL were elevated, resulting in significant increases of the OPG/RANKL ratio. Statistically insignificant and less stimulation on RANKL

expression together with the efficient expression of OPG by soluble Si led to more distinct increase of the OPG/RANKL ratio than DW. There is accumulated evidence [26–28,49] that the high concentration of Ca ions in the microenvironment of bone-resorbing sites [52] stimulates the expression of both RANKL and OPG mRNAs in osteoblast/stromal cells and regulates both osteoblastogenesis and osteoclastogenesis. In the present study, DW seemed to be more effective in osteoblastogenesis than soluble Si whereas the latter was important in antiosteoclastogenesis.

In conclusion, although its mechanism needs further investigation, the additions of either soluble Si or deep-sea water, from which NaCl was eliminated, to the culture medium or to the diet, stimulated the $^{45}CaCl_2$ uptake in both osteoblasts and osteoclast-like cells or enhanced the osteoblastogenesis- and osteoclastogenesis-related gene expressions in the mouse bone marrow, leading to the improvement of biochemical and mechanical properties of bone in mice.

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