Milk Basic Protein Facilitates Increased Bone Mass in Growing Mice

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Summary Milk basic protein (MBP) comprises a group of basic whey proteins and is effective in preventing bone loss by promoting bone deposition (bone formation) and suppressing withdrawn (bone resorption). We previously revealed the bone protective effects of MBP during life phases involving excessive bone resorption, such as in adults and postmenopausal women, and in animal models (ovariectomized rats and mice). However, it was unclear whether MBP increases bone mass during the growth stage, when there is more bone formation than resorption. We therefore investigated the effect of MBP supplementation on bone mass in 6-wk-old mice provided water supplemented with MBP [0.01%, 0.1%, 1.0% (w/w)] or deionized water (control) ad libitum for 10 wk. Analysis by micro-computerized tomography showed that MBP significantly increased tibia cortical bone mineral density and femur trabecular bone volume to tissue volume compared with mice provided deionized water. Next, the function of MBP in bone remodeling (bone formation and resorption) was evaluated using an in vitro system and the results demonstrated that MBP directly promoted osteoblast proliferation and inhibited osteoclastogenesis. Moreover, the plasma level of insulin-like growth factor-1 was increased by MBP supplementation, suggesting that MBP indirectly promoted osteoblast proliferation/differentiation. These effects enhance bone formation and/or inhibit bone resorption, resulting in increased bone mass in growing mice.

Key Words MBP, milk, bone, whey protein

Bone mass [bone mineral density (BMD) and bone strength] increase during the growth stage of humans, and the maximum BMD, indicated as peak bone mass, is achieved at around age 30. Bone mass decreases thereafter with age, and excessive bone loss leads to osteoporosis. The magnitude of peak bone mass is considered a potential predictor for the onset of osteoporosis later in life (1) and thus attempts to increase bone mass during the growth stage may help reduce the risk of osteoporosis.

Milk is a good source of calcium, an essential component of bone. It is generally accepted that milk intake contributes to the bone remodeling process because of its physiological role in promoting skeletal growth in newborn animals. In addition to their high nutritional value, milk proteins such as casein and whey protein significantly affect bone health (2–5). We previously demonstrated in both in vitro and in vivo studies that milk whey protein promotes bone formation and inhibits bone resorption (6–9). Moreover, milk basic protein (MBP), a group of basic whey proteins isolated by cation exchange chromatography, prevents bone loss in ovariectomized animals (10–12), as well as in adults (13, 14) and postmenopausal women (15, 16). These studies revealed that MBP supplementation prevents osteoporotic bone loss by suppressing the decrease in bone mass, resulting in normalized bone turnover markers. However, it is unclear whether MBP facilitates an increase in bone mass during the growth stage, during which there is more bone formation than bone resorption. It is well known that the increase in bone mass during the growth stage is influenced by hormones. For example, insulin-like growth factor (IGF)-1, which mediates the action of growth hormone (GH), is a signaling compound that promotes osteoblast proliferation and inhibited osteoclastogenesis. Moreover, the plasma level of insulin-like growth factor-1 was increased by MBP supplementation, suggesting that MBP indirectly promotes osteoblast proliferation/differentiation. These effects enhance bone formation and/or inhibit bone resorption, resulting in increased bone mass in growing mice.

MATERIALS AND METHODS

Preparation of milk basic protein. Milk basic protein was prepared according to the method described by Toba et al. (10). In brief, fresh milk was defatted by
centrifugation and loaded onto a sulfonated chitopearl resin (Fuji-Bousaki, Tokyo, Japan) cation exchange column. The loaded column was washed with deionized water, and the adsorbed proteins were eluted with 1.0 M sodium chloride. The purified MBP fraction was dialyzed in a cellulose membrane tube (Sanko Junyaku, Tokyo, Japan) and freeze-dried. The protein concentration was ~98% and the powdered MBP contained several minor components.

Animal experiment design. Five-week-old male C3H/HeJ mice (CLEA Japan, Inc., Tokyo, Japan) were fed a commercial chow (CE-2; CLEA Japan, Inc.) for 1 wk. The mice were divided into four groups (n=10) with no significant difference in body weight and given free access to MBP-containing water [0.01%, 0.1%, 1.0% (w/w)] or deionized water (control group) for 10 wk. All mice were fed an AIN-76 (American Institute of Nutrition 1977) diet ad libitum during the 10-wk experimental period. Body weight was measured once a week, and both food and water intake were measured every 2 d. The daily MBP intake was calculated from the water intake and the concentration of MBP in the water available to each mouse. Food efficiency was calculated from the body weight gain and food intake of each mouse over the entire experimental period. All mice were housed individually in stainless-steel cages under temperature- and humidity-controlled conditions (23°C and 40±5% relative humidity) with a 12 h light/dark cycle. The mice were treated in accordance with the animal experimentation regulation of the Milk Science Research Institute of Megmilk Snow Brand, which are based on the guidelines proposed by the Science Council of Japan. The experimental protocol was registered under the number 20131210 Growing C3H mice No. 04.

Micro-computerized tomography analysis of bone. Bones were analyzed using micro-computerized tomography performed using an R-mCT (Rigaku, Tokyo, Japan). The left tibias were scanned once a week during the 10-wk experimental period at 90 kV, 150 μA, and 10 magnification under isoflurane-induced anesthesia (Mylan Seiyaku, Tokyo, Japan). The cortical BMD (mg cm⁻³) of each tibia was measured in the diaphyseal region extending 3.63 mm in the proximal direction from the joint of the tibia to the fibula. The right femur removed from mice after the 10-wk experimental period was scanned using the same conditions as described above. The femur trabecular bone volume to tissue volume (BV/TV,%)) and BMD were analyzed at the secondary spongiosa extending 0.2 mm from the proximal tip of the primary spongiosa. BMD and BV/TV were determined using 3D bone morphology software (TRI/3D-BON; Ratoc, Tokyo, Japan).

Biomarkers in plasma. At the end of the experimental period, the mice were anesthetized with isoflurane, and blood was obtained from the abdominal inferior vena cava using syringe needles rinsed with ethylene-diamine-tetra-acetic acid (EDTA; Wako Pure Chemical Industries, Ltd., Osaka, Japan). Plasma was collected by centrifugation at 3,000 × g at 4°C for 30 min and stored at −80°C until analysis. Plasma concentrations of the following markers were measured using commercial ELISA kits according to the manufacturer’s instructions: bone-specific alkaline phosphatase (BALP), Mouse Bone-specific Alkaline Phosphatase ELISA kit (Cusabio Biotech, Wilmington, DE); γ-carboxylated osteocalcin (Gla-OC), Mouse Gla-Osteocalcin High Sensitive EIA kit (Takara Bio, Otsu, Japan); tartrate-resistant acid phosphatase (TRAP) 5b, MouseTRAP™ Assay (Immunodiagnostic Systems, Boldon, UK); C-terminal fragments of type I collagen (CTX I), RatLaps™ EIA (Immunodiagnostic Systems); insulin-like growth factor (IGF)-1, Mouse IGF-1 HS ELISA (Immunodiagnostic Systems); desacyl-ghrelin, desacyl-ghrelin enzyme immunoassay kit (Bertin Pharma, Bretonneux, France); and acyl-ghrelin, acyl-ghrelin enzyme immunoassay kit (Bertin Pharma). For measuring the level of acyl-ghrelin, blood was collected by using syringe needles previously rinsed with EDTA, then placing the blood into tubes containing p-hydroxymercuribenzoic acid as an esterase inhibitor. Immediately after centrifugation at 3,000 × g for 10 min at 4°C, the collected plasma samples were acidified by adding one-tenth volume of 1 N HCl to prevent modification of octanoic acid.

Mouse osteoblastic MC3T3-E1 cell proliferation assay. Mouse osteoblast MC3T3-E1 cells were provided by Dr. H. Kodama (Tohoku Dental College, Fukushima, Japan), and a cell proliferation assay was performed as previously described (12). In brief, cells were suspended in α-modified Eagle’s medium (αMEM; Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone, South Logan, UT), 100 U mL⁻¹ penicillin and 0.1 mg mL⁻¹ streptomycin (αMEM/FBS), then seeded into 96-well plates at 4×10³ cells per well. Cells were cultured for 24 h at 37°C. The culture medium was then replaced with fresh serum-free medium containing MBP solution (0.001 to 0.1 mg mL⁻¹, dissolved in PBS) or PBS (used as negative control) and cultured for 24 h. Cells were labeled with 1×10⁻⁶ M 5-bromo-2'-deoxyuridine (BrDU) during the last 2 h of the 24 h incubation period. The incorporation of BrDU into DNA was detected using a cell proliferation enzyme-linked immunosorbent assay (GE Healthcare, Little Chalfont, UK). Absorbance at 450 nm was measured with a Wallac ARVO MX 1420 multi label counter (Perkin Elmer Japan, Kanagawa, Japan).

Primary calvarial osteoblast differentiation assays. Primary osteoblasts (POB) were obtained by sequential enzyme digestion of excised calvarial bone from 5-d-old C57BL/6J mice (CLEA Japan, Inc.) as follows. The excised calvarial bones were incubated with digestion solution [0.1% collagenase A (Roche Diagnostics K.K., Basel, Switzerland) and 0.2% dispase II (Roche Diagnostics K.K.) in PBS] for 10 min at 37°C, then the first digestion solution was discarded. Next, the calvarial bones were incubated in fresh digestion solution and this solution was collected. This step was repeated three more times, then the digestion solutions were pooled and passed through a 70 μm cell strainer (Becton, Dickinson & Co, Franklin Lakes, NJ). The filtrate con-
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In the absence or presence of 0.01 mg mL⁻¹ for 2 d to confluence. The cells were then incubated collected BMCs were re-suspended in EDTA (GIBCO) for 5 min at 37˚C and then seeded into confluence, the cells were collected using 0.25% trypsin-24-well plates at 1.5×10⁴ cells per well and cultured 3 d in osteogenic medium [αMEM/FBS containing POB was centrifuged at 420 g for 5 min. The cells were re-suspended in αMEM/FBS and cultured in 9-cm dishes (3–4 calvariae/dish) for 2 d at 37˚C. Upon confluence, the cells were collected using 0.25% trypsin-EDTA (GIBCO) for 5 min at 37˚C and then seeded into 24-well plates at 1.5×10⁴ cells per well and cultured for 2 d to confluence. The cells were then incubated in the absence or presence of 0.01 mg mL⁻¹ MBP in osteogenic medium [αMEM/FBS containing 1×10⁻⁸ M dexamethasone (Wako Pure Chemical Industries, Ltd.), 0.05 mg L⁻¹ ascorbic acid (Wako Pure Chemical Industries, Ltd.) and 1×10⁻⁶ M β-glycerophosphate (MERK Millipore, Darmstadt, Germany)]. The osteogenic medium was changed every 2 d. After 3, 9, or 16 d culture, the cells were fixed with 10% neutral phosphate-buffer and visualized with alkaline phosphatase (ALP) stain and Von Kossa stain.

In vitro co-culture osteoclastogenesis assay. The assay was conducted as described by Yasuda et al. (20) with some modifications. In brief, mouse marrow-derived stromal cell line ST2 cells, obtained from the RIKEN Cell Bank (Tsukuba, Japan), were seeded into 96-well collagen-coated plates at 2×10⁴ cells per well and precultured for 48 h. Bone marrow cells (BMCs) were collected from 7- to 8-wk-old male ddy strain mice (CLEA Japan, Inc.) by flushing the femurs with αMEM. The collected BMCs were re-suspended in αMEM/FBS, seeded at 2×10³ cells per well onto ST2 cell layers, and cultured for 24 h. The medium was removed and replaced with culture medium containing 1×10⁻⁸ M 1α,25-dihydroxy vitamin D (Sigma), and 1×10⁻⁷ M dexamethasone (Sigma), then medium containing MBP solution (0.001 to 0.1 mg mL⁻¹) or PBS (negative control) was added. The medium was replaced every 3 d with fresh medium containing 1α,25-(OH)₂D₃, dexamethasone, and MBP or PBS. After 6 d culture, the BMCs were washed with PBS and fixed with acetone-ethanol (1:1) for 1 min, then treated with p-nitrophenyl phosphate solution (1.5 mg mL⁻¹ p-nitrophenyl phosphate, 20 mM sodium tartaric acid, and 50 mM citric acid buffer, pH 4.5). The reaction was stopped by adding 1 mM sodium hydroxide, and tartrate-resistant acid phosphatase (TRAP) activity was evaluated by measuring the absorbance at 405 nm.

Table 1. Body weight gain, food intake and food efficiency for the duration of the experiment.¹

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>MBP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.01%</td>
<td>0.10%</td>
</tr>
<tr>
<td>Initial body weight (g)</td>
<td>21.88±0.14</td>
<td>21.91±1.31</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>35.02±2.60</td>
<td>34.11±1.95</td>
</tr>
<tr>
<td>Body weight gain (g/d)</td>
<td>0.18±0.04</td>
<td>0.17±0.02</td>
</tr>
<tr>
<td>Food intake (g/d)</td>
<td>4.06±0.17</td>
<td>4.03±0.36</td>
</tr>
<tr>
<td>MBP intake (mg/d)</td>
<td>—</td>
<td>0.87±0.10</td>
</tr>
<tr>
<td>Food efficiency²</td>
<td>0.046±0.009</td>
<td>0.042±0.005</td>
</tr>
</tbody>
</table>

¹ Results are expressed as the mean±SD, control and 1.0% MBP, n=7; 0.01% MBP and 0.1% MBP, n=10.
² Food efficiency was determined using the formula: food efficiency = body weight gain (g)/70 (d)/food intake (g/d).

Mouse bone marrow osteoclast differentiation assays. Bone marrow macrophages (BMMs) derived from 6-wk-old male C57B/6J mice (CLEA Japan, Inc.) were prepared as described previously (21). In brief, cells were collected by flushing the femurs and tibias with αMEM, then red blood cells were removed using an ammonium chloride solution. After washing, the cells were cultured in αMEM/FBS with 1% macrophage colony-stimulating factor (M-CSF) conditioned medium (M-CSF-CM) prepared as described by Yogo et al. (22). After 12 h, non-adhering cells were collected and cultured for a further 3 d in αMEM/FBS with 3% M-CSF-CM. The cells were collected, seeded into 24-well plates at 2×10⁴ cells per well, and incubated overnight. The medium was replaced with osteoclastogenic medium containing 500 ng mL⁻¹ of receptor activator NF-kappa-B ligand (RANKL) prepared as described by Miyamoto et al. (23), 30 ng mL⁻¹ M-CSF (R&D Systems, Minneapolis, MN), and MBP solution (0.001 to 0.1 mg mL⁻¹) or PBS (negative control). After 3 d culture, the cells were fixed with methanol and visualized with TRAP stain. TRAP-positive giant multinucleated (MN) cells were counted under a microscope.

Statistical analysis. Data are expressed as the mean and standard deviation. Statistical analysis was performed using Stat View® ver.5 software (SAS Institute Inc., Cary, NC). Significance was estimated by Tukey’s post hoc test. Differences in mean values between the groups were considered significant at p<0.05. Mice whose femurs were broken during excision were excluded from statistical analysis, so the final number of mice in the control and 1.0% MBP groups were both 7.

RESULTS

Body weight gain, food intake, and food efficiency

The body weight gain, food intake, and food efficiency of mice whose drinking water was supplemented with 0 (control), 0.01%, 0.1%, or 1.0% (w/w) MBP for 10 wk did not differ among the four groups (Table 1). The intake of MBP calculated from water intake by the 0.01% MBP, 0.1% MBP, and 1.0% MBP groups was 0.87, 7.07, and 95.62 mg d⁻¹, respectively. These results indicate that MBP supplementation had no influence on body weight gain, food intake, or food efficiency.
Effect of MBP on bone mineral density in growing mice

To evaluate the dose-dependent effect of MBP supplementation on bone mass, we measured the diaphyseal BMD of the tibia (which provides the cortical bone mass) and the metaphyseal BMD and BV/TV (which provide the trabecular bone mass). The diaphyseal BMD was higher in the MBP (0.01%, 0.1%, and 1.0%) groups than in the control group at every measurement point during the experimental period (Fig. 1). In particular, at the 5th and 7th week, there were significant differences among the MBP (0.01%, 0.1%, and 1.0%) groups and the control group. The metaphyseal BMD was significantly higher in the MBP (0.01%, 0.1%, and 1.0%) groups than in the control group at the 10th week (Table 2). However, no differences in metaphyseal BMD were observed among the four groups, perhaps due to simultaneous increases in both bone mineral content and BV. These results suggest that 0.01% MBP supplementation is sufficient to increase bone mass in growing mice.

Fig. 1. Effect of milk basic protein (MBP) on tibial cortical bone mineral density (BMD). Six-week-old C3H/HeJ mice were given to MBP-containing water [0.01%, 0.1%, 1.0% (w/w)] or deionized water (control group) for 10 wk. The tibias were scanned using micro-computerized tomography once a week during the 10-wk experimental period, after which BMD was determined. Values are expressed as the mean±SD: control, 7 mice; 0.01% MBP, 10 mice; 0.1% MBP, 10 mice; 1.0% MBP, 7 mice. Lower-case letters indicate significant difference by Tukey’s post hoc test, p<0.05: a, control vs. 0.01% MBP; b, control vs. 0.1% MBP; c, control vs. 1.0% MBP.

Table 2. Bone morphologic parameters of the excised femoral trabecular bone determined using micro-computerized tomography at the 10th week of the experiment.†

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>0.01%</th>
<th>0.10%</th>
<th>1.0%</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMD (mg cm⁻³)</td>
<td>907.99±20.06</td>
<td>910.29±16.00</td>
<td>919.25±17.96</td>
<td>912.01±8.48</td>
</tr>
<tr>
<td>BV/TV (%)</td>
<td>3.19±1.04</td>
<td>4.91±1.03</td>
<td>4.96±1.09</td>
<td>4.80±0.97</td>
</tr>
</tbody>
</table>

† Results are expressed as the mean±SD: control and 1.0% MBP, n=7; 0.01% MBP and 0.1% MBP, n=10.

a,b Different superscript letters indicate significant difference, p<0.05 with Tukey’s post hoc test.

MBP, milk basic protein; BMD, bone mineral density; BV, bone volume; TV, tissue volume.
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observed, indicating that MBP appears to affect osteoblast proliferation rather than osteoblast differentiation.

To evaluate whether MBP treatment affects osteoclastogenesis, ST2 cells (a type of immature osteoblast) and BMC (an osteoclast progenitor) were co-cultured. As shown in Fig. 4, MBP dose-dependently inhibited TRAP activity when the concentration of MBP was more than 0.01 mg mL\(^{-1}\), indicating that MBP inhibits osteoblast-mediated osteoclastogenesis. When BMM (an osteoclast progenitor) was stimulated with cytokines (RANKL and M-CSF), MBP dose-dependently inhibited the formation of TRAP-positive MN cells (mature osteoclasts) when the concentration of MBP was more than 0.01 mg mL\(^{-1}\) (Figs. 5A and 5B), indicating that MBP inhibits cytokine-induced osteoclastogenesis. These results suggest that MBP suppresses osteoclast differentiation directly, but does not influence osteoblast differentiation.

### DISCUSSION

In this study, we administered MBP to young, growing mice and found that mice provided drinking water supplemented with MBP show significantly increased cortical BMD and trabecular BV/TV levels compared with mice provided MBP-free water (Fig. 1 and Table 2). Higher peak bone mass due to increased bone mass during the growth stage is known to be effective in reducing the risk of osteoporosis (1). Thus, our results suggest that MBP supplementation in young individuals is beneficial for achieving higher peak bone mass and thus for maintaining a healthy skeleton throughout life.

No dose-dependent effect of MBP on cortical BMD was observed in the current study; however, the values

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**Table 3. Plasma biomarkers at the 10th week of the experiment, as determined by ELISA.**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Control</th>
<th>MBP 0.01%</th>
<th>MBP 0.1%</th>
<th>MBP 1.0%</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALP (µg/mL)</td>
<td>2.22±1.32</td>
<td>1.48±0.54</td>
<td>1.49±0.30</td>
<td>1.29±0.42</td>
</tr>
<tr>
<td>Gla-OC (ng/mL)</td>
<td>28.33±3.81</td>
<td>25.69±3.23</td>
<td>26.84±2.36</td>
<td>28.53±7.59</td>
</tr>
<tr>
<td>TRAP 5b (U/L)</td>
<td>5.82±0.49</td>
<td>5.46±0.56</td>
<td>5.66±0.27</td>
<td>5.48±0.72</td>
</tr>
<tr>
<td>CTX I (ng/mL)</td>
<td>14.51±6.10</td>
<td>17.27±5.98</td>
<td>13.28±3.82</td>
<td>12.38±2.20</td>
</tr>
<tr>
<td>IGF-1 (ng/mL)</td>
<td>303.69±31.72a</td>
<td>314.58±26.32a</td>
<td>343.52±43.27a</td>
<td>398.94±80.05b</td>
</tr>
<tr>
<td>Desacyl-ghrelin (pg/L)</td>
<td>351.60±128.06a</td>
<td>461.76±252.61a</td>
<td>494.23±286.92b</td>
<td>1042.95±655.62b</td>
</tr>
<tr>
<td>Acyl-ghrelin (pg/mL)</td>
<td>272.02±133.38</td>
<td>258.74±107.40</td>
<td>215.52±108.42</td>
<td>218.02±103.26</td>
</tr>
</tbody>
</table>

1 Results are expressed as the mean±SD: control and 1.0% MBP, n=7; 0.01% MBP and 0.1% MBP, n=10.

\(a,b\) Different superscript letters indicate significant difference, \(p<0.05\) with Tukey’s post hoc test.

MBP, milk basic protein; BALP, bone-specific alkaline phosphatase; Gla-OC, γ-carboxylated osteocalcin; TRAP 5b, tartrate resistant acid phosphate; CTX I, C-terminal fragments of type I collagen; IGF-1, insulin-like growth factor-1.
in mice provided MBP (0.01%, 0.1% and 1.0%) were higher than those in mice provided MBP-free water at every measurement point (Fig. 1), suggesting that 0.01% MBP (0.87 mg d⁻¹) is sufficient, and the effect on bone mass might be saturated at higher concentrations. In addition, at week 10, statistically significant difference in cortical BMD also disappeared. This result may due to the large deviation: nevertheless, there still had been a little increasing effect. In contrast, trabecular BV/TV in mice provided MBP was significantly higher compared to controls (Table 2), suggesting that the influence of MBP continued even at week 10. Another possibility was that, since this study was designed to provide MBP to healthy animals, bone mass could not increase drastically. To further investigate the efficacy and dose dependency of MBP on bone mass in growth stage, we will conduct a study in a future project, in which MBP is provided to malnourished animals, e.g., under low calcium absorption conditions.

The in vitro study revealed that MBP treatment promoted both the induction of osteoblast proliferation (Fig. 2) and the inhibition of osteoclastogenesis (Figs. 4 and 5); however, no changes in bone turnover markers were observed even when MBP supplementation resulted in altered bone mass. This suggested that the bone remodelling process is resistant to MBP supplementation in the growth stage. In other words, MBP supplementation does not cause any abnormality in the bones of healthy animals. There are some studies reporting that the administration of nutrients to growing animals results in the increase in bone mass but no change in bone turnover markers (5, 26, 27). Kelly et al. pointed out that studying the effect of nutrients on bone mass is difficult to perform because of the highly stable nature of bone, which makes the extremely small changes that occur very hard to detect, and the detection of such small changes requires several months (5).

Since the influence of MBP supplementation on bone mass is not strong, extension of the experimental period may be required to detect the changes in bone turnover markers.

A previous report showed that circulating IGF-1 is necessary for the proper acquisition of peak bone mass (28). Sims et al. indicated that growth hormone receptor knockout mice, which exhibit impaired bone growth due to a reduction in the number of osteoblasts, were rescued by treatment with IGF-1 (17). Therefore, an increase in serum IGF-1 level by MBP supplementation may have the ability to increase in bone mass by promoting osteoblast-mediated bone formation. Osteoblasts and osteoclasts are both reported to express IGF-1 receptor, and IGF-1 knockout mice showed a marked reduction in number and differentiation of both osteoblasts and osteoclasts, leading to osteopenia (29–31). In an in vitro study, IGF-1 was shown to be required for maintaining the normal interaction between osteoblasts and osteoclasts to support osteoclastogenesis through its regulation of RANKL and RANK expression (32, 33). Although MBP treatment markedly suppressed osteoclastogenesis (Figs. 4 and 5), remarkable suppression of bone resorption markers was not observed (TRAP 5b and CTX I in Table 3). This might be because MBP supplementation exerted simultaneously conflicting functions on osteoclastogenesis: direct inhibitory effect of MBP and indirect promotive effect of IGF-1. On the other hand, MBP supplementation may accelerate osteoblast-mediated bone formation synergistically via both a direct promotive effect of MBP and an indirect one of IGF-1 on osteoblast proliferation/differentiation.

We previously obtained insights into the mechanism
of IGF-1 production by MBP supplementation by revealing that MBP affects ghrelin secretion (34). Ghrelin, an endogenous ligand for GH secretagogue receptor, is primarily synthesized in the stomach (35) and exists in the peripheral circulation in two forms: n-octanoylated at the serine-3 residue (acyl-ghrelin), and non-acylated (desacyl-ghrelin). In this study, we found that the plasma level of desacyl-ghrelin was significantly higher in mice provided water supplemented with 1.0% MBP compared to mice provided MBP-free water, consistent with the increase in circulating IGF-1 (Table 3). Ghrelin acts as a GH secretagogue, directly promotes osteoblast proliferation, and indirectly promotes osteoblastic bone formation synergistically via both direct (induction of osteoblast proliferation) and indirect (promotion of ghrelin production and/or IGF-1 production mediated osteoblast proliferation) mechanisms, and that these effects of MBP supplementation lead to increased bone formation in the growth stage, where there is more bone formation than resorption.

In conclusion, MBP supplementation increases bone mass in growing mice due to the promotion of osteoblast-mediated bone formation and/or the inhibition of osteoclast-mediated bone resorption.

Acknowledgments
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