Oral bovine lactoferrin improves bone status of ovariectomized mice

Anne Blais,2 Arnaud Malet,1,2,3 Takashi Mikogami,3 Christine Martin-Rouas,4 and Daniel Tomé1,2
1 Institut National de la Recherche and 2 AgroParisTech, Centre de Recherche et Nutrition Humaine de l’Île de France, UMR914 Nutrition Physiology and ingestive Behavior, F-75005 Paris; 3 Armor Protéines, Le Pont, F-35460 Saint-Brice-en-Cogés; and 4 Groupe Soparind Bongrain, F-78220 Viroflay, France

Submitted 21 November 2008; accepted in final form 14 March 2009

Blais A, Malet A, Mikogami T, Martin-Rouas C, Tomé D. Oral bovine lactoferrin improves bone status of ovariectomized mice. Am J Physiol Endocrinol Metab 296: E1281–E1288, 2009. First published March 31, 2009; doi:10.1152/ajpendo.90938.2008.—The aim of the present study was to evaluate the effect of dietary lactoferrin on bone metabolism in vivo using a postmenopausal animal model. We investigated whether bovine lactoferrin (bLF) ingestion could prevent loss in ovariectomized mice. Twelve-week-old female C3H mice either ovariectomized or sham operated were fed for 27 wk with the control diet (AIN-93M with 140 g of total milk protein as a protein source per kg of diet). Four groups of ovariectomized mice received diets including different concentrations of bLF (1, 5, 10, or 20 g of total milk protein were replaced by bLF). Ovariectomy induced a decreased uterine weight and a smaller gain of bone mineral density. Immunoreactive bLF was detected in the peripheral blood, and its concentration was related to the amount of bLF ingestion. bLF supplementation to the diet improved bone mineral density (BMD) and femoral failure load in a dose-dependent manner. We confirmed the direct effects of bLF in vitro using established and primary cultures of murine bone cells. Addition of bLF to the culture medium at a concentration of between 1 and 1,000 µg/ml stimulated both cell growth and differentiation of osteoblastic MC3T3 cells while inhibiting the growth of preosteoclastic RAW 267.4 cells. In primary culture of mixed bone cells, an enhanced osteoblast differentiation was associated with an inhibition of osteoclast differentiation at lower bLF concentrations (1–10 µg/ml). In conclusion, these findings suggest that dietary lactoferrin supplementation can have a beneficial effect on postmenopausal bone loss by modulating bone formation and resorption.

Osteoporosis is one of the most critical disorders occurring in postmenopausal women. It is characterized by a reduced bone mineral density (BMD) and an increased risk of fracture. The postmenopausal bone loss is a consequence of estrogen deficiency that increases osteoclast activity (37), leading to an imbalance between bone formation and bone resorption. Estrogens play a fundamental role in skeletal growth and bone metabolism. It has been recognized that in response to estrogen deficiency, osteoclastogenesis occurs (39). TNF is one of the cytokines responsible for the augmented osteoclastogenesis (47). Ovariectomy increased T cell TNF production, which increased macrophage colony-stimulating factor-induced and receptor activator of NF-κB ligand (RANKL)-induced osteoclastogenesis (8). Moreover, the presence of increased levels of TNF was reported in the bone marrow of ovariectomized (Ovx) animals and in blood cells of postmenopausal women (38, 44). Postmenopausal osteoporosis should be also regarded as the result of an inflammatory process (52). Recent animal studies demonstrated that estrogen deficiency causes bone loss by mechanisms associated with inflammatory and oxidative processes (13, 23, 34). Recent studies suggest a strong relationship between bones and the immune system; this interface should play a role not only in the regulation of inflammatory bone turnover but also in animal models of postmenopausal osteoporosis and in basal regulation of bone homeostasis (52).

Interestingly, lactoferrin (LF), an 80-kDa iron-binding glycoprotein of the transferrin family, has been demonstrated to inhibit in vitro osteoclast-mediated bone resorption (31). LF was also demonstrated to have in vitro anabolic, differentiating, and antiapoptotic effects on osteoblasts and to inhibit osteoclastogenesis (8). Moreover, in vivo local injection of LF above the hemicalvaria increases bone formation and bone area in adult mice. LF is found in milk secreted by the mammary gland but also in tears, synovial fluids, saliva, seminal fluid, and, to a lesser extent, the specific granules of neutrophils (27, 29). LF has a role in host nonspecific defense (9, 12, 14, 25, 42). This property is related to its ability to either sequester iron in biological fluids or destabilize the membranes of microbes, and this plays a direct antimicrobial role in secretion and at the surface of epithelia by limiting the proliferation and adhesion of microorganisms. In addition to its direct antimicrobial effects, LF is believed to modulate the inflammatory process mainly by preventing the release of inflammatory cytokines that induce recruitment and activation of immune cells at inflammatory sites (26). Moreover, LF’s biological functions are dependent on its target cells and might be related to its capacity to bind to various molecules in the cells.

The present study addresses the bone action of bovine LF (bLF) in vitro and in vivo. We used Ovx mice as an animal model of postmenopausal osteoporosis to study the effect of dietary bLF supplementation on bone status. In vitro studies were performed using established cell lines and an original primary cell culture system, allowing the growth of both differentiated osteoblasts and osteoclasts. The results support the potential of oral LF supplementation to improve postmenopausal bone loss.

MATERIALS AND METHODS

LF preparation. bLF was isolated from fresh skimmed milk by dual cation exchange chromatography. Briefly, fresh skimmed bovine milk was passed through a sulphopropyl-type ion exchanger SPEC 70 (Pall Biosepra) at 4°C, and the bound proteins were eluted with 1.7 M NaCl. Demineralized eluted protein fraction was reapplied to the cation exchanger S Sepharose Fast Flow (GE Healthcare) at 4°C, and the bound proteins were eluted in steps with 0.5 M NaCl at pH 6.5 and 0.9 M NaCl at pH 8.5. The latter fraction containing LF was demineralized, microfiltered, and spray-dried. bLF purity of the final product was >98%, as assessed by reversed-phase HPLC using VYDAC 214TP54 (Grace).

http://www.ajpendo.org 0193-1849/09 $8.00 Copyright © 2009 the American Physiological Society E1281
Ovx mice model. Female 6-wk-old C3H/HeN strain mice (n = 48) were purchased from Harlan. They were housed in a room controlled for temperature (22 ± 1°C) under a 12:12-h light-dark cycle and were given free access to a standard-pellet diet and water. All experimental procedures used during these experiments complied with institutional guidelines and policies to prevent pain and distress under license from the French Veterinary Service (A75-05-19). Forty-two 12-wk-old female C3H mice were Ovx, and eight mice were sham operated (Sham). Mice were anesthetized with ketamine (100 mg/kg) + xylazine (10 mg/kg), and morphine was given to avoid pain. One week after surgery, Ovx mice were divided into five groups of eight animals and fed for 27 wk with either the control diet, AIM-93M, including 140 g of total milk protein/kg of diet (Ovx C), or with a diet in which the total milk protein content had been adjusted to allow the incorporation of 1 (Ovx 1), 5 (Ovx 5), 10 (Ovx 10), or 20 (Ovx 20) g/kg bLF to the diet. Diet composition is shown in Table 1. At weeks 5, 9, 13, 17, and 27 after surgery, at the beginning of the light cycle, the blood of each mouse was collected from the orbital sinus to evaluate immunoreactive bLF concentrations.

Bone mineral density, biomechanical strength of femurs, and calcium contents of femora. Radiographic dual-energy X-ray absorptiometry analysis using a Lunar Piximus densitometer (GE Medical Systems, software version 1.4× lunar) was performed to determine the entire body, lumbar, and femoral BMD and bone mineral content (BMC). The BMD of the entire body, lumbar spines, and right femoral bone of each mouse was measured, under anesthesia, 1, 5, 9, 13, 17, 22, and 27 wk after the ovarectomy. Through whole body scanning, the BMD and BMC of the entire body, lumbar spine, and right femur were analyzed.

The right femur of each mouse was cleaned from muscle, preserved in a tube, and kept on ice until the three-point bending test was performed a few hours later. Three-point bending evaluates the elastic and plastic properties contributing to femoral strength. Yield load, which is a measurement of the femoral elastic limit, was determined as the point where the slope of the load-deformation curve deviates from being a straight line. The peak load is a measure of the maximum force that the femur withstands before fracture. This test was carried out at the femoral midpoint, where cortical bone is predominant. The biomechanical strength testing was determined using a digital calibrator (Texturometer TA XT2i using expert exceed version 07.13; Cedarlane Laboratories, Hornby, ON, Canada). Femurs were positioned such that the posterior side was placed on two base supports, with the midpoint directly under the cross-head. The cross-head was lowered at a constant speed of 0.1 mm/s until fracture occurred. The peak load and yield load were calculated from the load-displacement curve.

The left femur of each mouse was cleaned from muscle and dried overnight at 100°C, weighed, and then ashed at 550°C for 48 h. The ashes were extracted with 1 ml of 1 M HCl. The amount of calcium in femurs was determined by atomic absorption spectrophotometry using a Zeeman polarized spectrophotometer (Hitachi Z-5000).

Cell line cultures. The osteoblastic cell line MC3T3-E1, obtained from the European Collection of Cell Cultures, was cultured at 37°C under a 5% CO2-95% air atmosphere in petri dishes with α-MEM (Invitrogen France) supplemented with 10% fetal bovine serum (FBS) (HyClone; Perbio). Cells were subcultured every 5 days using 0.05% trypsin and 0.54 mM EDTA in Ca2+/Mg2+-free PBS. For the experiment, cells were seeded onto 48-well plates (IWAKI; ATCG) at a density of 5 X 104 cells/cm2. LF was added to the culture medium 48 h after seeding, at final concentrations of between 1 and 1,000 μg/ml. Cell growth after 72 h of incubation in the presence of LF was evaluated using a FluorReporter Blue Fluorometric dsDNA Quantitation kit (Molecular Probes). The fluorometric method we used has been developed to count adherent cells in the range of 1,000–100,000 cells/well for proliferation studies. In a first experiment, cells were counted and compared with DNA quantification. The standard curve obtained was not significantly different at 5, 10, or 14 days of culture. For the DNA quantification, cells were rinsed with PBS and frozen at −80°C until DNA quantification. On the day of the determination, cells were unfrozen in water and homogenized, one half of the cell homogenate was used for DNA quantification, and the other half was used to evaluate alkaline phosphatase (ALP) activity as a marker of bone formation (43). To evaluate ALP activity we used a fluorescent substrate, 4-methylumbelliferyl phosphate (Sigma), and the activity was expressed as a function of DNA content.

The preosteoclastic RAW 264.7 monocyte/macrophage cell line, obtained from the European Collection of Cell Cultures, was cultured at 37°C under a 5% CO2-95% air atmosphere in petri dishes with DMEM (Invitrogen) supplemented with 10% FBS (HyClone; Perbio). For the experiments, cells were seeded onto 48-well plates (IWAKI) at a density of 5 X 104 cells/cm2. Forty-eight hours after seeding, bLF was added to the culture medium at final concentrations of between 1 and 1,000 μg/ml. Cell growth after 72 h of incubation in the presence of LF was evaluated using a FluoReporter Blue Fluorometric dsDNA Quantitation kit.

Primary culture of murine bone cells. BALB/c mice were euthanized by decapitation, and tibias were removed aseptically. Bones

Table 1. Dietary composition

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Control</th>
<th>Ovx 1</th>
<th>Ovx 5</th>
<th>Ovx 10</th>
<th>Ovx 20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cow’s milk protein*</td>
<td>140</td>
<td>139</td>
<td>135</td>
<td>130</td>
<td>120</td>
</tr>
<tr>
<td>Bovine lactoferrin*</td>
<td>0</td>
<td>1</td>
<td>5</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>Corn starch</td>
<td>622.4</td>
<td>622.4</td>
<td>622.4</td>
<td>622.4</td>
<td>622.4</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100.3</td>
<td>100.3</td>
<td>100.3</td>
<td>100.3</td>
<td>100.3</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>o-Cellulose</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>AIN 93 M mineral mixture†</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>AIN 93 M vitamin mixture†</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Choline</td>
<td>2.3</td>
<td>2.3</td>
<td>2.3</td>
<td>2.3</td>
<td>2.3</td>
</tr>
</tbody>
</table>

*Armor Proteines, Saint-Brice-en-Coglès, France; ¯Cerestar, Haubourdin, France; ¯Eurosure, Paris, France; ¯Baillly, Aulnay-sous-Bois, France; ¯Medias Filtrants Durieux, Marne-la-Vallée, France (Alphacell, ICD Pharmaceuticals, Orsay, France); ¯ICN Pharmaceuticals, Orsay, France.

†Ovx 1, 5, 10, and 20: 1, 5, 10, and 20 g/kg bovine lactoferrin, respectively.

Downloaded from http://ajpendo.physiology.org/ on 2019-06-29 by 10.220.32.246
were placed in a petri dish containing α-MEM that included 10% FBS. Bones were broken with a scalpel, and bone marrow was scraped out. Bone pieces and the medium with the cells were collected in a tube and vigorously shaken. The solution was filtered through a 70-μm cell strainer. Cells were collected by centrifugation for 5 min at 800 g and resuspended in α-MEM with 10% FBS and 10⁻⁸ M 1α,25-dihydroxyvitamin D₃. Cells obtained from four to five tibias were plated in one 75-cm² flask. After 6 days of culture, the cells were scraped, collected, counted, and resuspended in α-MEM with 10% FBS and 10⁻⁸ M 1α,25-dihydroxyvitamin D₃ and seeded in petri dishes at a density of 2.5 × 10⁴ cells/cm². BSA or bLF was added to the culture medium 24 h after seeding. Cells were incubated for 14 days in the presence of BSA or bLF and then washed with PBS, fixed, and stained with Giemsa (Sigma). Osteoclast-like multinucleated cells that express specifically tartrate-resistant acid phosphatase (TRAP) were evaluated using the method described by Sakai et al. (41). DNA quantification and ALP activity were determined as described in Cell line cultures. Morphological observation was done by light microscopy with ×100 magnification to look at the effect of LF on cell growth.

The BD BioCoat Osteologic bone cell culture system (BD Biosciences) was used to characterize and measure osteoclast-mediated bone resorption. The mixed bone cell suspension prepared as described above was seeded at a density of 4 × 10⁴ cells/cm². BSA (final concentration 1,000 μg/ml) or bLF (final concentration 10, 100, or 1,000 μg/ml) was added to the culture medium 24 h after seeding. Cells were incubated for 14 days in the presence of BSA or bLF. The digestion of submicron synthetic calcium phosphate thin films was observed with a light microscope with ×10 magnification to evaluate cell resorption activity.

Statistical analysis. Results are expressed as means ± SD. Statistical analyses were performed using SAS software. Results were compared using a one-way analysis of variance to assess the effect of diets. Significance was established at P < 0.05.

RESULTS

Body composition and blood LF concentration of Ovx mice fed LF-supplemented diet. Effectiveness of the Ovx procedure was confirmed by the reduction in uterine weight of Ovx mice compared with Sham mice (Sham 114 ± 41 mg vs. Ovx 67 ± 29 mg, P < 0.001). However, LF supplementation did not significantly modify uterine weight.

At the end of the experiment, after 27 wk, there were no significant differences in body weight between the different mice groups (Fig. 1), although Ovx mice tended to gain more weight than the Sham mice. Indeed, carcass, WAT, and subcutaneous fat weight of Ovx mice were significantly heavier compared with Sham mice. Both WAT and carcass increased, but the body composition (WAT/carcass) of Ovx mice was significantly increased compared with Sham mice (Sham 0.70 ± 0.12 vs. Ovx 0.84 ± 0.012, P < 0.05). Addition of bLF to the diet did not significantly modify any parameter.

Immunoreactive bLF (iLF) concentration in individual mouse peripheral blood was evaluated after bLF ingestion for 4, 8, 12, 16, and 26 wk. The iLF blood concentrations of mice receiving the bLF-supplemented diets for 8 wk were significantly increased compared to those receiving the control diet (Fig. 2) and were correlated to the bLF concentration in the diet (r = 0.998). Similar results were obtained after 4, 12, 16, and 26 wk (data not shown).

LF-supplemented diet improved BMD and bone mechanical properties of Ovx mice. Total, femoral, and lumbar BMD were evaluated every month during the 6 mo of the experiment. The total BMD of Sham mice was significantly higher (P < 0.05) than that of Ovx mice fed the control diet (Ovx C) from week 5 to week 27 postsurgery (Fig. 3A). Moreover, Fig. 3 shows that the ovariectomy delayed the progress of BMD since the Ovx C mice reached the maximal BMD at about week 17 postsurgery vs. week 9 postsurgery for the Sham mice.

bLF supplementation restored a reduced gain of total BMD in Ovx mice compared with the Sham mice (Fig. 3B). A significant increase in the total BMD compared with Ovx C was reported 9 wk after surgery for Ovx 10 and Ovx 20 and at week 17 and week 27 for Ovx 5, Ovx 10, and Ovx 20. The Ovx 20 total BMD at week 27 was even higher than that of Sham mice.

After 26 wk of bLF supplementation, BMD was also evaluated at the femoral (Fig. 4A) and lumbar levels (Fig. 4B). Femoral and lumbar BMD values measured in Ovx mice were smaller compared with Sham mice values. bLF supplementation restored BMD at both femoral and lumbar level. At the femoral level, a significant increase of the BMD compared with Ovx C was observed for Ovx 5, Ovx 10, and Ovx 20. Moreover, bLF supplementation at 20 mg/kg of diet also significantly increased femoral BMD compared with the Sham mice. At the lumbar level, a significant increase of the BMD compared with Ovx C was observed only for Ovx 10 and Ovx 20. bLF supplementation appeared to be more efficient at the femoral level. Figure 4D also shows femoral calcium content at week 27 postsurgery. bLF supplementation increased femoral calcium content of Ovx mice in a dose-dependent manner, and a significant increase was observed for Ovx 5, Ovx 10, and Ovx 20.

Biomechanical properties of femur were also evaluated at 26 wk after bLF supplementation (Fig. 4C). Because the primary effects of estrogen deficiency on bone biomechanical properties were on stiffness (yield load) and failure load (peak load) (20), both were measured. Yield load and peak load of Ovx C mice were significantly reduced compared with those of Sham mice. bLF supplementation to the diet increased both parameters compared with Ovx C. In contrast with what was ob-
We then developed a primary culture of murine bone cells. After 14 days of culture, the presence of two cell types was observed in the culture, i.e., round-shaped, osteoclast-like, multinuclear giant cells that express TRAP and smaller osteoblast-like mononuclear cells (Fig. 6A). The proportion of TRAP-positive cells was never more than 5%. Figure 6B shows that when mixed primary culture of murine bone cells was grown in the presence of a low concentration of bLF (10 \( \mu \text{g/ml} \)), the number of cells increased. However, Fig. 6C and D, show that higher concentration of bLF (100 and 1,000 \( \mu \text{g/ml} \)) decreased the number of cells. To evaluate the effect of bLF on growth of mixed primary cells, DNA was quantified. We observed that as bLF concentration in the culture medium increased, the number of osteoclasts decreased. Indeed, no multinucleated cells were observed at 1,000 \( \mu \text{g/ml} \), indicating a complete inhibition of osteoclast differentiation (Fig. 6D). Moreover, at this concentration, the cells seeded in the plates attached but never developed, the number of cells remaining the same during the next 14 days. DNA quantification confirmed that low concentration of bLF significantly increased cell growth but a concentration of 100 or 1,000 \( \mu \text{g/ml} \) inhibited cell growth (Fig. 7). We report a strong dose-dependent effect of bLF on primary culture of murine bone cells. The cell growth increase was coupled to a significant increase in ALP activity at the lower bLF concentrations (1 and 10 \( \mu \text{g/ml} \)). Osteoclast numeration showed that bLF at concentrations ranging from 1 to 1,000 \( \mu \text{g/ml} \) inhibited osteoclast differentiation (Fig. 7). The higher bLF concentrations (100 and 1,000 \( \mu \text{g/ml} \))...
has been shown to depend on both the genetic background and developmental stages. Moreover, bone loss after ovariectomy is different, leading to various bone loss patterns, according to the animal model used. In this C3H mouse model the ovariectomy was performed at 12 wk, when BMD was still increasing. Osteoporosis is the consequence of an inadequate bone formation to compensate for the increased bone resorption associated with estrogen deficiency. In this study, we used in vivo and in vitro models to evaluate the effect of oral LF on bone status. The results showed that oral LF supplementation dose-dependently improves BMD and femoral failure load of Ovx mice. Both direct action through increased blood concentration and indirect action of LF by modulation of the immune system are probably involved in both enhanced osteoblastic activity and inhibition of osteoclastic activity.

In the present study, C3H-Ovx mice or sham-operated mice were used as an in vivo animal model of postmenopausal osteoporosis (11, 50). In this C3H mouse model the ovariectomy was performed at 12 wk, when BMD was still increasing. Accordingly, we observed a reduced rate of bone mass gain. These results are in accordance with recent studies showing that in mice bone cell sensitivity to estrogen deficiency is different, leading to various bone loss patterns, according to the developmental stages. Moreover, bone loss after ovariectomy has been shown to depend on both the genetic background and the anatomic location of bone growth, which is a factor that determines the magnitude of the loss in strength after ovariectomy. Cortical bone mechanical properties are typically determined by whole bone mechanical tests. In this study, whole femur was subjected to three-point bending and stiffness and failure load measurements, which have been shown to be the most sensitive indicators of changes in bone morphology (20). Although bone mechanical tests do not exactly mimic in vivo loading, these tests provide quantitative assessments of how mechanical behavior is affected by the change in morphological architecture of the bone after ovariectomy. Considering that anatomic location of bone loss is a factor that determines bone strength, further microarchitecture analyses should help us to better identify where and how LF modulates bone metabolism and to understand LF mechanism of action on bones.

The lowest LF concentration used (1 g/kg of diet) did not significantly modify any of the parameters tested except femoral failure load. Indeed, maximum break and yield loads in Ovx 1 were significantly improved for femurs compared with those of Ovx C. The morphology of long bone is different from that of short bone, such as vertebrae, and this may explain why different effects on femur and vertebrae were reported in the present study.

Both in vivo and in vitro experiments suggested that the improvement of bone metabolism by LF given orally could partly result from a direct local action of LF on bone. Evaluation of blood-immunoreactive LF showed that dietary LF absorption into peripheral blood was directly related to LF content in the diet and that LF blood concentration remained at the same level from 4 to 26 wk of LF ingestion. Moreover, we observed that the peripheral LF blood concentrations went significantly inhibited growth of both osteoblasts and osteoclasts.

To better characterize the effect of bLF on osteoclast activity, primary culture of murine bone cells was performed for 14 days on the BD BioCoat Osteologic bone cell culture system. This allowed the measurement of osteoclast-mediated bone resorption. Osteoclast activity was shown by digestion of submicron synthetic calcium phosphate thin films; digestion of a wide surface of synthetic films was observed when cells were grown without bLF in the presence of BSA (Fig. 8). The digestion was drastically diminished in the presence of bLF at concentrations of 10 and 100 µg/ml (Fig. 8, B and C) and completely abolished at 1,000 µg/ml (Fig. 8D). These results indicate that bLF inhibits osteoclast differentiation and its resorption activity at a physiologically occurring concentration.

**DISCUSSION**

Osteoporosis is the consequence of an inadequate bone formation to compensate for the increased bone resorption associated with estrogen deficiency. In this study, we used in vivo and in vitro models to evaluate the effect of oral LF on bone status. The results showed that oral bLF supplementation dose-dependently improves BMD and femoral failure load of Ovx mice. Both direct action through increased blood concentration and indirect action of LF by modulation of the immune system are probably involved in both enhanced osteoblastic activity and inhibition of osteoclastic activity.

In the present study, C3H-Ovx mice or sham-operated mice were used as an in vivo animal model of postmenopausal osteoporosis (11, 50). In this C3H mouse model the ovariectomy was performed at 12 wk, when BMD was still increasing. Accordingly, we observed a reduced rate of bone mass gain. These results are in accordance with recent studies showing that in mice bone cell sensitivity to estrogen deficiency is different, leading to various bone loss patterns, according to the developmental stages. Moreover, bone loss after ovariectomy has been shown to depend on both the genetic background and the skeletal site (1, 4, 19, 21, 30, 36, 53, 54, 59). In the mouse model, bLF-supplemented diet improved BMD and bone mechanical properties after ovariectomy. The lowest bLF concentration tested was effective at maintaining femur mechanical properties but did not have any significant effect on the calcium content or BMD. Biomechanical principles dictate the anatomic location of bone growth, which is a factor that determines the magnitude of the loss in strength after ovariectomy. Cortical bone mechanical properties are typically determined by whole bone mechanical tests. In this study, whole femur was subjected to three-point bending and stiffness and failure load measurements, which have been shown to be the most sensitive indicators of changes in bone morphology (20). Although bone mechanical tests do not exactly mimic in vivo loading, these tests provide quantitative assessments of how mechanical behavior is affected by the change in morphological architecture of the bone after ovariectomy. Considering that anatomic location of bone loss is a factor that determines bone strength, further microarchitecture analyses should help us to better identify where and how LF modulates bone metabolism and to understand LF mechanism of action on bones.

The lowest LF concentration used (1 g/kg of diet) did not significantly modify any of the parameters tested except femoral failure load. Indeed, maximum break and yield loads in Ovx 1 were significantly improved for femurs compared with those of Ovx C. The morphology of long bone is different from that of short bone, such as vertebrae, and this may explain why different effects on femur and vertebrae were reported in the present study.

Both in vivo and in vitro experiments suggested that the improvement of bone metabolism by LF given orally could partly result from a direct local action of LF on bone. Evaluation of blood-immunoreactive LF showed that dietary LF absorption into peripheral blood was directly related to LF content in the diet and that LF blood concentration remained at the same level from 4 to 26 wk of LF ingestion. Moreover, we observed that the peripheral LF blood concentrations went

```

Fig. 4. Femur mineral density (A), lumbar mineral density (B), mechanical properties of the right femur, maximum break load (white bars) and yield load (black bars) (C), and calcium content of the left femur (D) after 26-wk ingestion of the experimental diets. Ovx mice were fed for 26 wk with Ovx C or the diet supplemented with Ovx 1, Ovx 5, Ovx 10, or Ovx 20 bLF. The Sham mice were fed for 26 wk with the control diet. Values are expressed as means ± SD; n = 8. Values with different letters are significantly different (P < 0.05).
```
back rapidly to control level when the LF supplementation was stopped. This strongly suggests that the immunoreactive bLF measured in peripheral blood is derived from the diet. In a previous study (10), we have shown that intact immunoreactive bLF is absorbed from mouse intestine into the blood and subsequently localized within various tissues. LF resistance to proteolytic digestion (22, 51) and the presence of LF receptor in the mouse intestinal brush border (17) may explain the rapid bLF uptake from the lumen to the blood (15, 18). Moreover, our in vitro experiments demonstrated that LF could directly act on bone cells. bLF at low physiological concentrations (5 μg/ml) stimulated osteoblastic MC3T3-E1 cell growth and can

![Fig. 5. A: effect of bLF on proliferation of MC3T3 cells (○) and RAW 267.4 cells (■). B: alkaline phosphatase activity of MC3T3 cells. DNA content quantification was used to evaluate cell growth in the presence of bLF for 3 days. Alkaline phosphatase activity was evaluated as function of DNA content in the presence of bLF for 3 days. The values are expressed as a stimulation index. Data are means ± SD of 3 determinations done on 3 different cultures. *Significant difference from control (P < 0.05).](image)

![Fig. 6. Effect of bLF on primary culture of murine bone cells. Cells obtained from tibias were grown in standard petri dishes for 14 days in presence of BSA (A) or bLF at concentrations of 10 (B), 100 (C), or 1,000 μg/ml (D). Cells were fixed and stained with Giemsa. Photographs were taken at the same magnification (×10).](image)

![Fig. 7. Effect of bLF on proliferation of primary culture of murine bone cells. DNA content quantification was used to evaluate murine bone cell growth in the presence of bLF for 14 days. The values are expressed as a stimulation index. Data are means ± SD of 3 determinations done on 4 different cultures. *Effect of bLF on alkaline phosphatase activity of primary culture of murine bone cells. Alkaline phosphatase activity was evaluated as function of DNA content for cells grown in the presence of B. The values are expressed as a stimulation index. Data are means ± SD of 3 determinations done on 4 different cultures (P < 0.05). **Effect of bLF on osteoclast differentiation of primary culture of murine bone cells. The number of differentiated osteoclasts, designated as cells with 3 or more nuclei, was counted after 14 days of culture in the presence of bLF. The values are expressed as inhibition index compared with control. Values are means ± SD of 3 determinations done on 4 different cultures. *Significant difference from control (P < 0.05).](image)

![Fig. 8. Primary culture of murine bone cells was grown for 14 days on BD BioCoat Osteologic bone cell culture system to characterize and measure osteoclast-mediated bone resorption shown by digestion of submicron synthetic calcium phosphate thin films. Cells were grown in the presence of BSA (A) or bLF at concentrations of 10 (B), 100 (C), and 1,000 μg/ml (D). Photographs were taken at the same magnification (×10).](image)
stimulate growth of osteoblast and inhibit osteoclastogenesis in primary culture of murine bone cells. A more important stimulation of cell growth was obtained in the presence of 100 µg/ml LF. In addition, osteoblast differentiation increased gradually up to a LF concentration equal to 1,000 µg/ml. bLF at a concentration ranging from 10 to 1,000 µg/ml was found to inhibit RAW cell growth. As previously reported, LF action on osteoclasts is strikingly different since it produces an important arrest of osteoclastogenesis (8, 31). These results were confirmed in mixed primary culture of murine bone cells. At high concentration, growth and differentiation of both osteoclasts and osteoblasts were completely arrested, a phenomenon that has been correlated to a decreased expression of NF-κB (8). However, in agreement with Cornish et al. (8), at low physiological concentrations, LF exerted a dual effect characterized by an important inhibition of osteoclast differentiation with a stimulating effect on osteoblast proliferation. The transcription factor NF-κB is known to play a central role on the regulation of inflammatory and immune responses and on the control of cell mitosis and apoptosis (45). In opposition to the studies of Conish et al. (8), Oh et al. (35) reported in neutrophils an activation of NF-κB by LF concentration ranging from 20 to 100 µg/ml. This suggests that LF can trigger different pathways, depending on the target cells.

LF is also presumed to have an indirect action on bone metabolism. An increasing number of studies indicates that LF modulates inflammatory processes and antioxidant activity mainly by preventing the release of cytokines that induce recruitment and activation of immune cells at inflammatory sites (26). It is also possible that LF regulates bone homeostasis through the modulation of cytokine production. One of the mechanisms responsible for ovariectomy-induced bone loss is a cytokine-driven increase in osteoclast formation (7, 51). Osteoclast differentiation takes place when bone marrow macrophages are costimulated by the two osteoclastogenic factors, i.e., RANKL and the macrophage colony-stimulating factor (MCSF) (7). In an estrogen-deficient situation (such as in postmenopausal women and Ovx animals), TNFα upregulates osteoclast formation (39, 51) by stimulating the production of RANKL and MCSF and causes bone loss in rodents and human (6, 39). Thus, we propose that dietary intake of bLF can have an indirect effect on bone through its capacity to regulate the immune system by decreasing TNF production. In agreement with this hypothesis, bLF oral administration has been shown to suppress TNFα production, to increase IL-10 production in adjuvant-stimulated arthritic rats (16), and to lower the expression of TNFα in intestinal lymphocytes of healthy mice (46). Thus, dietary supplementation of bLF to Ovx mice would decrease TNF production, which subsequently normalized the elevated osteoclastogenesis observed in estrogen-deficient situations.

Interestingly, other milk whey protein fractions have been demonstrated to have an effect on bone resorption. Several human studies have confirmed the beneficial effects of the basic protein fraction from bovine milk on bone metabolism (2, 3, 48, 57). Furthermore, in vitro studies have demonstrated that high-mobility group-like protein (47) and kininogen fragment 1.2 (56, 58), which are found in the basic protein fraction of bovine milk, promote osteoblast proliferation. Cystatin C (32) and angiogenin (33), two proteins that are found in milk basic protein fraction, are known to act as inhibitory factors on osteoclastic bone resorption. Whether LF is an active component in these basic protein fractions remains to be demonstrated.

In conclusion, the results obtained in the present study with Ovx mice show the effectiveness of dietary bLF supplementation on postmenopausal bone loss by modulating bone formation and resorption. The demonstration of the dietary bLF transfer into peripheral blood in an immunoreactive form and the dual effects of bLF on osteoblasts and osteoclasts support a direct action of bLF on bone cells. Moreover, the involvement of indirect actions of ingested bLF via the modulation of cytokine production remains to be proven.

Altogether, our data suggest that dietary bLF supplementation may represent a preventive strategy for bone disorders in our experimental model. The question of the relevance of such a strategy for postmenopausal bone disorder treatment in humans requires clinical investigations.

ACKNOWLEDGMENTS

We thank Caroline Vierra and Stéphane Besançon for technical assistance in determination of BMD, femoral biomechanical strength, and calcium content.

GRANTS

A. Malet received a CIFRE grant from the French Ministry of Higher Education and Research.

REFERENCES


