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Milk contains several components effective for bone health. In the previous *in vitro* and *in vivo* studies, we have shown that milk whey protein, especially its basic protein fraction (milk basic protein [MBP]), promoted bone formation and suppressed bone resorption. This present study examines the effect of MBP on the biochemical markers of bone metabolism in healthy adult men. Experimental beverages containing MBP (300 mg of MBP a day) were given to 30 normal healthy adult men for 16 days. The serum osteocalcin concentration had increased significantly after 16 days of ingesting the experimental beverage containing MBP. Urinary cross-linked N-teleopeptides of type-I collagen (NTx) excretion had decreased significantly after 16 days of ingesting MBP. The urinary NTx excretion was related to the serum osteocalcin concentration after 16 days of ingestion. These results suggest that MBP promoted bone formation and suppressed bone resorption, while maintaining the balance of bone remodeling.

Key words: milk basic protein; bone formation; bone resorption; remodeling; healthy adult men

Ever since the dairy cow was domesticated by the ancient Egyptians in 4000 B. C., milk has been making a major contribution to human health. In particular, milk is recommended for bone health because it is an excellent calcium source and contains several profitable components for calcium absorption in the intestines such as lactose and phosphopeptides formed by the proteolytic digestion of milk casein.[1-5] Several dietary factors such as vitamin K_2 and isoflavone,[6,7] which directly affect bone metabolism (bone formation and resorption), have recently been widely noticed. Milk is also considered to directly affect bone metabolism because it has a functional role in the growth of newborn animals and is an excellent source of nutrients for human health. We have been searching for biologically active components in milk that could stimulate bone formation and/or suppress bone resorption.

We have found that milk whey protein (a by-product of the cheese-making process) stimulated the proliferation and differentiation of osteoblastic cells. The active factors stimulating the osteoblastic cells were found to be concentrated in the basic protein fraction (milk basic protein [MBP]).[8] We have also reported that MBP digested by gastrointestinal enzymes was absorbed through the small intestine and that digested MBP retained its stimulating activity for the proliferation and differentiation of osteoblastic cells.[8] We have also found that milk whey and MBP increased such bone protein as collagen and enhanced the bone-breaking energy in young ovariectomized rats.[9-11] We have drawn the conclusion from our previous *in vitro* and *in vivo* studies that milk whey protein and MBP have direct and/or indirect effects on bone formation. In a study with an unfractionated bone cell culture system and isolated osteoclasts, we found that milk whey and MBP suppressed osteoclast-mediated bone resorption.[12,13] We have reported that MBP digested by gastrointestinal

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Abbreviations: MBP, milk basic protein; PICP, procollagen I carboxy-terminal propeptide; NTx, cross-linked N-teleopeptides of type-I collagen; BMD, bone mineral density; Cr, creatinine

enzymes was absorbed through the small intestine and that digested MBP retained its suppressive activity against bone resorption in the study based on the unfractionated bone cell culture system.[12] Moreover, we have found that MBP suppressed osteoclast-mediated bone resorption and prevented bone loss in aged ovariectomized rats.[13] We have drawn the conclusion from our previous *in vitro* and *in vivo* studies that milk whey protein and MBP have direct and/or indirect effects on bone resorption.

In our previous controlled trial of the effect of MBP supplementation (40 mg of MBP a day) on the bone mineral density (BMD) in healthy adult women for six months, we found that MBP supplementation increased BMD and suppressed the urinary excretion of cross-linked N-teleopeptides of type-I collagen (NTx; a biochemical marker for bone resorption).[14] We are thus attempting to clearly find the effect of MBP on bone formation from such bone formation markers as serum osteocalcin. The purpose of this present study is to examine the effect of MBP on the biochemical markers of bone metabolism in healthy adult men when slightly more MBP (300 mg of MBP a day) was ingested.

Subjects and Methods

Subjects. Thirty healthy men (mean ± SD age, 36.2 ± 8.5) were recruited through direct mailing and attending presentations about this study in our institute. The protocol was approved by the ethical committee of the participating institution. Written informed consent was obtained from each subject. The study complied with the code of ethics of the World Medical Association (Helsinki Declaration of 1964 as revised in 1989).

Study design and supplements. Thirty men received an experimental beverage containing MBP (300 mg of MBP a day). The beverage also contained an acidifier, sweetener, and flavor to provide a pleasant taste for the volunteers. MBP was obtained from fresh bovine milk which was defatted by centrifugation and loaded into a column packed with cation-exchange resin. The column was sufficiently washed with deionized water, and the bound protein was eluted with 1M NaCl. MBP was obtained by desalinizing and drying. The protein content of the MBP sample was 98% (w/w). The subjects were instructed to drink the beverage daily within any two hours for 16 days, and were advised to maintain their usual diets. Each received a physical checkup every week, and was subjected to urine and blood measurements before and after the 16 days of ingestion.

Analytic methods. Blood was drawn from 9 to 11 a.m. after the subjects had fasted for at least eight hours. Second spontaneous urine samples were collected from 9 and 10 a.m. before breakfast. Aliquots of these samples were frozen at −20°C until needed for analysis. Serum osteocalcin was measured by an immunoradiometric assay (BGP IRMA, Mitsubishi Kagaku, Tokyo, Japan), and serum procollagen I carboxy-terminal propeptide (PICP) was measured by a radioimmunoassay (Orion Diagnostica, Oulunsalo, Finland). Urinary cross-linked N-teleopeptides of type-I collagen (NTx) were measured by an enzyme-linked immunosorbent assay (Osteomark, Ostex International, Seattle, WA). All biochemical markers of bone metabolism were analyzed by Mitsubishi Bio-Clinical Laboratories (Tokyo, Japan). Calcium in the serum and urine was respectively analyzed by models 7450 and 7070 clinical analyzers (Hitachi, Tokyo). The urinary biomarkers were adjusted for creatinine (Cr) excretion and were given as per mmol Cr.

Statistical analysis. The results for the serum and urinary biomarkers were analyzed by Student's *t*-test for paired data to examine the difference before and after 16 days of ingesting the experimental beverage containing MBP. The correlation coefficient between the urinary NTx excretion and the serum osteocalcin concentration was tested with a single linear regression analysis, differences being considered significant if $P<0.05$. All calculations were performed by the GLM procedure in the SAS statistical analysis package,[15] and all tests were two-tailed.

Results

Characteristics of the subjects. Table 1 presents the subjects' characteristics. There was no significant difference before and after 16 days of ingestion in either of the parameters for weight and body mass index.

Biochemistry. The serum calcium level and urinary calcium excretion were unchanged after 16 days of ingesting the experimental beverage containing MBP (Table 2). The serum osteocalcin concentration had increased significantly after 16 days of ingestion (Table 2, $P<0.0001$), while the serum PICP level tended to have increased after 16 days of ingestion, although the difference was not significant (Table 2, $P=0.0872$). The urinary NTx excretion had decreased significantly after 16 days of ingestion (Table 2, $P<0.0001$). Individual changes in the serum osteocalcin concentration and urinary NTx excretion before and after 16 days of ingestion are shown in Fig. 1, an increased serum osteocalcin concentration being found in twenty-eight (93%) of the 30 subjects, and decreased of urinary NTx excretion being found in twenty-four (80%) of the 30 subjects. Figure 2 presents the correlation between the urinary NTx excretion and the serum osteocalcin concentra-

Table 1. Characteristics of the Subjects before and after 16 Days of MBP Ingestion[a]

	Before	After 16 days	Paired t-test, P value
Age (y)	36.2±8.5	—	—
Height (cm)	171.2±4.2	—	—
Weight (kg)	67.3±8.5	67.3±8.9	0.8299
BMI (kg/m^2)[b]	22.9±2.5	23.0±2.6	0.5839

[a] The results for the subjects' characteristics were analyzed by Student's t-test for paired data to examine the difference before and after 16 days of ingesting an experimental beverage containing MBP (milk basic protein). Each value is the mean±SD (n=30). Differences are considered significant if $P<0.05$.
[b] BMI, body mass index

Table 2. Biochemical Markers of the Subjects before and after 16 Days of MBP Ingestion[a]

	Before	After 16 days	Paired t-test, P value
Serum			
Calcium (mmol/l)	2.3±0.1	2.3±0.1	0.4820
Osteocalcin (ng/ml)	3.7±1.8	5.4±1.8	<0.0001
PICP[b] (ng/ml)	122.3±37.0	130.0±44.1	0.0872
Urine			
Calcium (mmol/mmol Cr[b])	0.21±0.11	0.23±0.10	0.3535
NTx[b] (nmol/mmol Cr)	31.5±10.2	26.8±9.6	<0.0001

[a] The results for the biochemical markers were analyzed by Student's t-test for paired data to examine the difference before and after 16 days of ingesting an experimental beverage containing MBP (milk basic protein). Each value is the mean±SD (n=30). Differences are considered significant if $P<0.05$.
[b] PICP, procollagen I carboxy-terminal propeptide; NTx, cross-linked N-teleopeptides of type-I collagen; Cr, creatinine

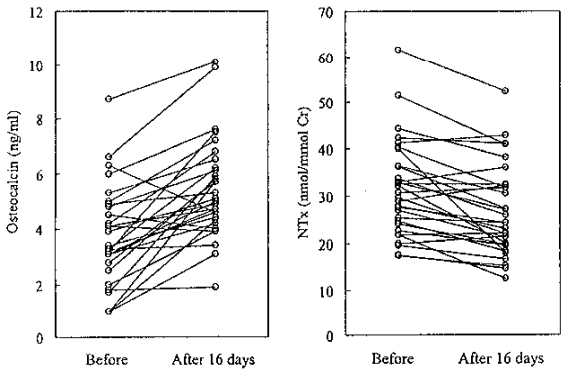

Fig. 1. Individual Changes in the Serum Osteocalcin Concentrations (left) and Urinary NTx Excretion (right) before and after 16 Days of Ingesting an Experimental Beverage Containing MBP.
NTx, cross-linked N-teleopeptides of type I collagen; MBP, milk basic protein

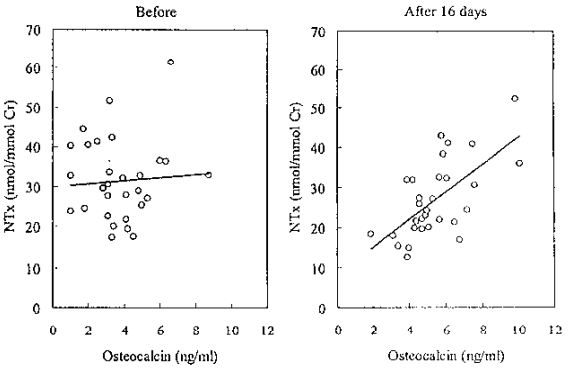

Fig. 2. Relationship between the Urinary NTx Excretion and the Serum Osteocalcin Concentration before (left) and after 16 Days (right) of Ingesting an Experimental Beverage Containing MBP.
The correlation coefficient before ingestion was 0.0641 ($P=0.7366$), and after 16 days was 0.6457 ($P<0.0001$). Differences are considered significant if $P<0.05$. NTx, cross-linked N-teleopeptides of type-I collagen; MBP, milk basic protein

tion. The urinary NTx excretion was not found to be related to the serum osteocalcin concentration before ingestion, but the urinary NTx excretion was found to be related to the serum osteocalcin concentration after 16 days of ingesting the experimental beverage containing MBP, with a correlation coefficient of 0.6457 ($P<0.0001$).

Discussion

A biochemical marker of bone turnover that reflects bone changes faster than BMD is available for measuring serum or urine. We measured serum osteocalcin and serum PICP as the biochemical markers of bone formation, because proteins released from osteoblasts, including osteocalcin and procollagen peptides, can be used to assess bone formation. The products of collagen breakdown, including collagen cross-links, can be used to assess bone resorption. We measured the urinary NTx excretion as the biochemical marker of bone resorption because NTx is reportedly more sensitive to a change in bone metabolism than deoxypyridinoline is.[16] In this study, we found that MBP supplementation increased the serum osteocalcin concentration and serum PICP and decreased the urinary NTx excretion in healthy adult men. These results suggest that MBP

promoted bone formation and suppressed bone resorption.

We have demonstrated in previous *in vitro* and animal studies, that MBP promoted bone formation by activating osteoblasts and suppressed bone resorption by its direct and/or indirect effects on osteoclasts.[8-13] Our results from the present human study are consistent with those from *in vitro* and animal studies. In the recent human study, we found that MBP supplementation (40 mg of MBP a day) increased BMD and suppressed the urinary excretion of NTx.[14] Our results about the effect of MBP on bone resorption from the present human study are consistent with those from the previous human study; however, we failed to clearly find an effect from MBP on the serum osteocalcin concentration. When slightly more MBP (300 mg of MBP a day) was ingested in the present human study, we found an increased serum osteocalcin concentration. The present human study indicates that the increased levels of BMD might have been caused by the promoting effect of MBP on bone formation and by its suppressing effect on bone resorption.

Bones are continuously undergoing a remodeling process through repeated cycles of destruction and rebuilding.[17] In healthy young adults, the amount of new bone formation approximately balances the amount of bone resorption. As we age, however, the balance shifts to favor bone resorption, which can result in debilitating diseases such as osteoporosis. Efforts to treat bone diseases have been primarily concentrated on the development of drugs to block bone resorption that decreases the formation or activity of osteoclasts.[18] To prevent bone diseases, it might be questionable to strongly block bone resorption because this will unbalance bone remodeling. It is important to investigate whether MBP actually causes a loss in the balance of bone remodeling because it has a suppressive effect on bone resorption. In the present study, the urinary NTx excretion (a biochemical marker of bone resorption) was not found to be related to the serum osteocalcin concentration (a biochemical marker of bone formation) before ingestion, but the urinary NTx excretion was found to be related to it after 16 days of ingestion. These results indicated that the subjects who had a higher activity of bone formation also had a higher activity of bone resorption after 16 days of ingestion. This phenomenon suggests that, while MBP suppressed bone resorption, it did not block bone resorption by bone remodeling. We consider that MBP promoted bone formation and suppressed bone resorption while maintaining the balance of bone remodeling.

MBP, which has basic isoelectric points, is believed to contain an array of profitable factors.[19] We have previously demonstrated that the active components in MBP related to bone formation were high mobility group-like protein and kininogen fragment 1·2 by a bioassay using osteoblastic MC3T3-E1.[20,21] Again, we found that milk cystatin purified from milk suppressed osteoclast-mediated bone resorption (data not shown). It is known that cathepsin, a protease secreted by osteoclasts, is responsible for bone resorption. It has been reported that cystatin C inhibited cathepsin as a protease inhibitor.[22-24] We thus consider that milk cystatin in MBP is one of the possible components that prevents bone resorption by inhibiting cathepsin. MBP is itself complex: it is a polyvalent fraction containing many profitable factors. Its effect on bone health is likely to be more than can be accounted for by any single constituent, and the totality of MBP's effect may be more than the sum of the parts. MBP might maintain the balance of bone remodeling because it contains several effective components for both formation and resorption.

In conclusion, our results suggest that MBP promoted bone formation and suppresses bone resorption in healthy adult men, and that it affected bone metabolism while maintaining a balance of bone remodeling. We believe that MBP might become a novel, natural, and desirable nutritional supplement for bone health.

References

1) Wong, N. P., and LaCroix, D. E., Biological availability of calcium in dairy products. *Nutr. Rep. Intern.*, **21**, 673-680 (1980).
2) Toba, Y., Kato, K., Takada, Y., Tanaka, M., Nakano, T., Aoki, T., and Aoe, S., Bioavailability of milk micellar calcium phospate-phosphopeptide complex in rats. *J. Nutr. Sci. Vitaminol.*, **45**, 311-323 (1999).
3) Wasserman, R. H., Lactose-stimulated intestinal absorption of calcium: a theory. *Nature*, **201**, 997-999 (1964).
4) Lee, Y. S., Noguchi, T., and Naito, H. Phosphopeptides and soluble calcium in the small intestine of rats given a casein diet. *Br. J. Nutr.*, **43**, 457-467 (1980).
5) Toba, Y., Takada, Y., Tanaka, M., and Aoe, S., Comparison of the effects of milk components and calcium source on calcium bioavailability in growing male rats. *Nutr. Res.*, **19**, 449-459 (1999).
6) Shiraki, M., Shiraki, Y., Aoki, C., and Miura, M., Vitamin K_2 (menatetrenone) effectively prevents fractures and sustains lumbar bone mineral density in osteoporosis. *J. Bone Miner. Res.*, **15**, 515-521 (2000).
7) Potter, S. M., Baum, J. A., Teng, H., Stillman, R. J., Shay, N. F., and Erdman, J. W., Soy protein and isoflavones: their effects on blood lipids and bone density in postmenopausal women. *Am. J. Clin. Nutr.*, [Suppl.] **68**, 1375S-1379S (1998).
8) Takada, Y., Aoe, S., and Kumegawa, M., Whey protein stimulates the proliferation and differentiation of osteoblastic MC3T3-E1 cells. *Biochem. Biophys. Res. Commun.*, **223**, 445-449 (1996).
9) Takada, Y., Kobayashi, N., Kato, K., Matsuyama, H., Yahiro, M., and Aoe, S., Effect of whey protein

on calcium and bone metabolism in ovariectomized rats. *J. Nutr. Sci. Vitaminol.*, **43**, 199–210 (1997).
10) Takada, Y., Matsuyama, H., Kato, K., Kobayashi, N., Yamamura, J., Yahiro, M., and Aoe, S., Milk whey protein enhances the bone breaking force in ovariectomized rats. *Nutr. Res.*, **17**, 1709–1720 (1997).
11) Kato, K., Toba, Y., Matsuyama, H., Yamamura, J., Matsuoka, Y., Kawakami, H., Itabashi, A., Kumegawa, M., Aoe, S., and Takada, Y., Milk basic protein enhances the bone strength in ovariectomized rats. *J. Food Biochemistry*, **24**, 467–476 (2000).
12) Takada, Y., Kobayashi, N., Matsuyama, H., Kato, K., Yamamura, J., Yahiro, M., and Aoe, S., Whey protein suppresses the osteoclast-mediated bone resorption and osteoclast cell formation. *Int. Dairy Journal*, **7**, 821–825 (1997).
13) Toba, Y., Takada, Y., Yamamura, J., Tanaka, M., Matsuoka, Y., Kawakami, H., Itabashi, A., Aoe, S., and Kumegawa M., Milk basic protein: a novel protective function of milk against osteoporosis. *Bone*, **27**, 403–408 (2000).
14) Aoe, S., Toba, Y., Yamamura, J., Kawakami, H., Yahiro, Kumegawa, M., Itabashi, A., and Takada, Y., Controlled trial of the effects of milk basic protein (MBP) supplementation on bone metabolism in healthy adult women. *Biosci. Biotechnol. Biochem.* **65**, 913–918 (2001).
15) SAS Institute Inc. SAS/STAT User's Guide, release 6.03, ed. Cary, NC. SAS Institute.
16) Hanson, D. A., Weis, M. A., Bollen, A. M., Maslan, S. L., Singer, F. R., and Eyre, D. R., A specific immunoassay for monitoring human bone resorption: quantitation of type I collagen cross-linked N-telopeptides in urine. *J. Bone Miner. Res.*, **7**, 1251–1258 (1992).
17) Ott, S. M., Theoretical and methodological approach. In "Principles of Bone Biology," eds. Bilezikian, J. P., Raisz, L. G., and Rodan, G. A., Academic Press, California, pp. 231–241 (1996).
18) Rodan, G. A., and Martin, T. J., Therapeutic approaches to bone diseases. *Science*, **289**, 1508–1514 (2000).
19) Francis, G. L., Regester, H. A., Webb, H. A., and Ballard, F. J., Extraction from cheese whey by cation-exchange chromatography of factors that stimulate the growth of mammalian cells. *J. Dairy Sci.*, **78**, 1209–1218 (1995).
20) Yamamura, J., Takada, Y., Goto, M., Kumegawa, M., and Aoe, S., High mobility group-like protein in bovine milk stimulates the proliferation of osteoblastic MC3T3-E1 cells. *Biochem. Biophys. Res. Commun.*, **261**, 113–117 (1999).
21) Yamamura, J., Takada, Y., Goto, M., Kumegawa, M., and Aoe, S., Bovine milk kininogen fragment 1-2 promotes the proliferation of osteoblastic MC3T3-E1 cells. *Biochem. Biophys. Res. Commun.*, **269**, 628–632 (2000).
22) Lerner, U. H., and Grubb, A., Human cystatin C, a cysteine proteinase inhibitor, inhibits bone resorption *in vitro* stimulated by parathyroid hormone and parathyroid hormone-related peptide of malignancy. *J. Bone Miner. Res.*, 7, 433–440 (1992).
23) Lerner, U. H., Johansson, L., Ransjö, M., Rosenquist, J. B., Reinholt, F. P., and Grubb, A., Cystatin C, an inhibitor of bone resorption produced by osteoblasts. *Acta Physiol. Scand.*, **161**, 81–92 (1997).
24) Morio, R., Yamaza, T., Nishiura, T., Nishimura, Y., Terada, Y., Abe, K., Himeno, M., and Tanaka, T., Immunocytochemical study of cathepsin L and rat salivary cystatin-3 in rat osteoclasts treated with E-64 *in vivo*. *Arch. Oral Biol.*, **42**, 305–315 (1997).