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Milk basic protein increases alveolar bone formation in rat experimental periodontitis

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Background and Objective: It is conceivable that the active components extracted from milk whey protein (i.e. milk basic protein, MBP) stimulate bone formation and suppress bone resorption. Periodontitis is characterized by excessive alveolar bone resorption. We examined whether milk basic protein could recover alveolar bone loss in rat experimental periodontitis.

Material and Methods: A nylon ligature was placed around the cervix of molars in 8-wk-old male Fischer rats for 20 d. Then, the ligature was removed and a powder diet containing 0.2 or 1.0% milk basic protein was provided daily for another 45–90 d. On days 45 and 90, the maxillae were extracted and analyzed using microcomputerized tomography (micro-CT), followed by histological analysis.

Results: Micro-CT images showed that alveolar bone resorption was severely induced around the molar by the 20-d ligature procedure. Treatment with high-dose milk basic protein (1.0%) clearly recovered ligature-induced alveolar bone resorption on days 45 and 90, whereas low-dose milk basic protein (0.2%) did not show such a clear effect. Histological examination clarified that the osteoid thickness of alveolar bone was dose dependently increased by milk basic protein treatment for 90 d.

Conclusion: These findings suggest that a systemic administration of milk basic protein may be effective for the recovery of alveolar bone loss in periodontitis.

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Alveolar bone resorption is a major problem in advanced-stage periodontitis. To recover bone loss, tissue regeneration therapy, using growth factors such as bone morphogenetic protein and fibroblast growth factor, have been examined in periodontitis patients. Although these agents have been topically applied to periodontal lesions, systemic agents for periodontal bone loss have not been actively developed.

Supplemental foods such as isoflavone, which increase bone formation, have also been studied for use in osteoporosis therapy (1). Milk whey protein, a by-product of cheese or casein manufacturing, affects bone metabolism in an anabolic manner and contains the active components of the basic protein fraction (i.e. milk basic protein) (2–6). Milk basic protein stimulates bone formation and suppresses bone resorption, as shown by *in vitro* and *in vivo* studies (5–7). It is also reported that supplementation with milk basic protein increased bone metabolism, even in healthy men and women (8,9). Moreover, when milk basic protein was administered to postmenopausal women for 6 mo, the bone mineral density of the lumbar vertebrae significantly increased (10).

Microcomputerized tomography (micro-CT) has been widely used for the study of bone metabolism in animals. Micro-CT can elaborate cross-sectional tomograms of ≈ 10 μm thick, and then build three-dimensional images via computer. Micro-CT analysis

has been used as a convenient method for the histomorphometrical study of long bones in ovariectomized rats and gene-deficient mice (11,12).

In the present study, we investigated the bone-formative effects of milk basic protein in rat experimental periodontitis by assessing morphological data obtained from micro-CT and histological sections.

Material and methods

Seventy male Fischer rats (8 wk of age) were housed in individual wire cages in a temperature- and humidity-controlled room (23 ± 1°C and 60 ± 5% relative humidity) with a 12-h light/dark cycle. The cervical area of the right second molar of the maxilla was ligatured with nylon thread (No. 5–0; Natsume, Tokyo, Japan) under anesthesia with sodium pentobarbital. After ligaturing, all rats were fed a control powder diet for 20 d. Then, 10 rats were killed and their maxillae were collected. These samples were defined as the control on day 0 and served as the baseline. In another 60 rats, the nylon thread was removed on day 0 and the rats were evenly divided into three groups of 20 rats per group, as follows: ligatured control group (control powder diet); MBP-L group (0.2% milk basic protein in powder diet); and MBP-H group (1.0% milk basic protein in powder diet). The rats were fed 15 g of diet per day for 45–90 d. The left maxillae without ligature were used as the non-ligatured control group.

Milk basic protein preparation

Milk basic protein was prepared according to previous reports (5–7). Milk basic protein was obtained from fresh bovine milk. The bovine milk was defatted by centrifugation. Acid whey was obtained from the defatted milk and loaded onto a column that was packed with 500 g of cation exchange resin (sulfonated chitopearl™; Fuji Bouseki, Tokyo, Japan). The collum was sufficiently washed with deionized water and the bound protein was eluted with 1 M NaCl. The milk basic protein obtained was freeze-dried, after dialyzing the eluted fraction in a cellulose membrane tube (Sanko-Junyaku, Tokyo, Japan).

Micro-CT analysis

Maxillae were scanned by micro-CT (Hitachi Medico, Tokyo, Japan). The CT was set as follows: pixel size, 1024 × 1024; slice thickness, 14 μm; magnification, 8×; voltage, 50 kV; and electrical current, 0.1 mA. The frontal sections were made parallel to the medial root of the second molar using a computer. The distance from the palatal cement–enamel junction to the alveolar bone crest was measured in the frontal section as a marker of bone height.

Histomorphometric analysis

For preparing undecalcified sections, the maxillae on day 90 were embedded in methylmethacrylate resin. The frontal sections, parallel to the medial root of the second molar, were prepared ≈20 μm thick. All samples were stained with Villanueva bone staining before embedding (13). All sections were observed under a light microscope at 100× and 400× magnifications. Osteoid thickness, which was stained dark violet, was measured.

Statistical analysis

All values in the figures are expressed as the mean ± standard deviation of six to 10 separate samples from each group. The significance between groups was estimated using one-way analysis of variance and Fischer's protected least significance test. p-Values of < 0.05 were considered significant.

Results

The images of micro-CT are shown in Fig. 1. An increase in palatal alveolar bone loss in the ligatured control group compared with the nonligatured control group was observed on day 0 (Fig. 1A,B). Similar alveolar bone loss in the ligatured control group was also identified on day 90 (Fig. 1C,D). This alveolar bone loss had been recovered in the MBP-H group by day 90, whereas it was not clear in the MBP-L group (Fig. 1E,F). In the nonligatured control, the distance from the cement-enamel junction to the alveolar bone crest was 266.3 ± 45.0 μm, 311.2 ± 49.2 μm and 303.4 ± 56.7 μm on days 0, 45 and 90, respectively (Fig. 2). This indicated that no significant change in length occurred during the experimental period. The distance in the ligatured control group was greater than in the nonligatured control; however, it did not change significantly with time (472.8 ± 51.0 μm, 527.2 ± 77.1 μm and 527.1 ± 62.3 μm on days 0, 45 and 90, respectively), indicating that the ligature-induced bone resorption continued for 90 days. In the MBP-L group, the distance showed no significant change compared with the ligatured control (486.7 ± 101.3 μm and 526.3 ± 80.0 μm on days 45 and 90, respectively). On the other hand, in the MBP-H group, the distance decreased significantly, compared with the ligatured control, on days 45 and 90, to 432.8 ± 47.8 μm and 407.3 ± 32.9 μm, respectively.

Villanueva bone staining on day 90 revealed that destruction of periodontal tissue, such as the rough surface of alveolar bone and irregular collagen fiber, was not observed in the ligatured control, MBP-L and MBP-H groups, with images similar to the nonligatured control observed (Fig. 3A–D). Alveolar bone loss was marked in the ligatured control and MBP-L groups, showing an increased distance from the cement-enamel junction to alveolar bone crest compared with the nonligatured control (Figs 3B,C). However, decreased alveolar bone loss was recognized in the MBP-H group (Fig. 3D). In highly magnified sections, osteoblasts appeared along the alveolar bone surface in all groups (Fig. 4, arrowheads). Osteoid, stained dark violet, appeared at the surface of alveolar bone beneath osteoblasts (Fig. 4, arrows). When the osteoid width was measured, increased osteoid thickness was observed in the MBP-L and MBP-H groups compared with nonligatured and ligatured controls. As shown

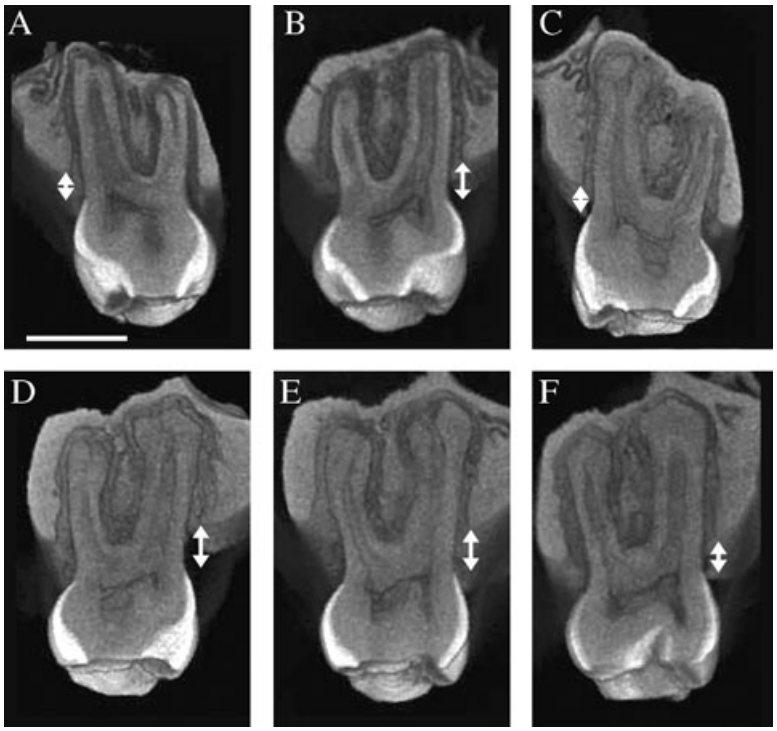

Fig. 1. Micro-CT images of the frontal sections of maxillary secondary molars from rats. (A) Day 0 in the nonligatured control; (B) day 0 in the ligatured control; (C) day 90 in the nonligatured control; (D) day 90 in the ligatured control; (E) day 90 in the MBP-L group; and (F) day 90 in the MBP-H group. Arrows show the distance from the cement–enamel junction to the alveolar bone crest (alveolar bone height). Bar, 1.0 mm. MBP-H group, rats receiving a high dose (1.0%) of milk basic protein in powder diet; MBP-L group, rats receiving a low dose (0.2%) of milk basic protein in powder diet.

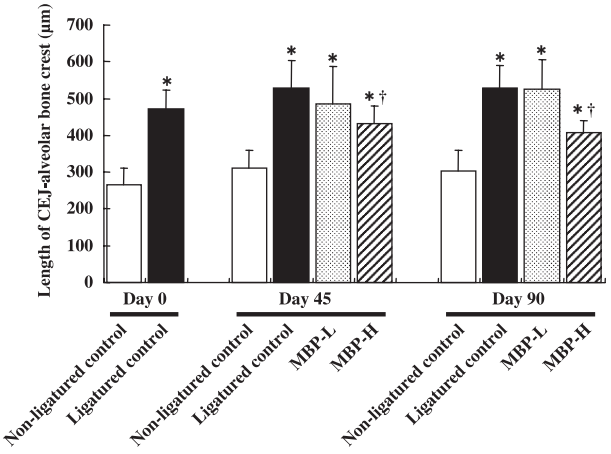

Fig. 2. Length from the cement–enamel junction (CEJ) to the alveolar bone crest as a marker of bone height in micro-CT. Data show the mean ± standard deviation from 10 rats in each group. $*p < 0.05$ compared with the nonligatured control. $\dagger p < 0.05$ compared with the ligatured control. MBP-H, rats receiving a high dose (1.0%) of milk basic protein in powder diet; MBP-L, rats receiving a low dose (0.2%) of milk basic protein in powder diet.

in Fig. 5, measurement of osteoid thickness revealed that the width increased in the ligatured control, MBP-L and MBP-H groups compared with the nonligatured control, and that the length in the MBP-L and MBP-H groups was higher than that in the ligatured control.

Discussion

Experimental animal models have been used to clarify the pathogenesis of periodontal diseases and develop new periodontal therapy (14,15). Ligatured methods have been accepted as useful experimental models of periodontitis with alveolar bone resorption (16,17). Lima *et al.* (18,19) reported that inflammatory changes in alveolar bone were induced by ligature placement and that inflammatory cells, including osteoclasts and lymphocytes, appeared beneath the ligature, and severe destruction of periodontal tissues was induced after 7 d. These data indicate that the ligature model is useful for evaluating bone loss in periodontitis. In this study, the placement of nylon thread rapidly induced alveolar bone loss (20 d), and bone loss was maintained for 90 d, even after removing the ligature. Using this experimental model, we were able to evaluate the effect of a bone-formative agent (milk basic protein) in periodontitis.

Micro-CT has been widely used in the study of bone metabolism. Previous reports showed that micro-CT is an effective method for histomorphometrical analysis of long bone in ovariectomized rats and gene-deficient mice (11,12,20). In dental science, including periodontology, few studies employ micro-CT, whereas micro-CT has been more commonly used in endodontic research (21,22). To date, analysis of X-ray photographs and histological sections of alveolar bone have been performed as conventional methods in periodontal research (23,24). As the periodontal tissue structure is complicated, many steps are necessary to produce histological sections. Micro-CT can produce accurate figures of hard periodontal tissues by constructing three-dimensional images via computer. In this study, we were able to obtain clear sections of the rat molar area using micro-CT, indicating that micro-CT is useful for analyzing alveolar bone.

Previous reports suggested that milk whey protein, containing milk basic protein, stimulated the proliferation and differentiation of osteoblastic MC3T3-E1 cells (3). It is believed, from a bioassay using osteoblastic

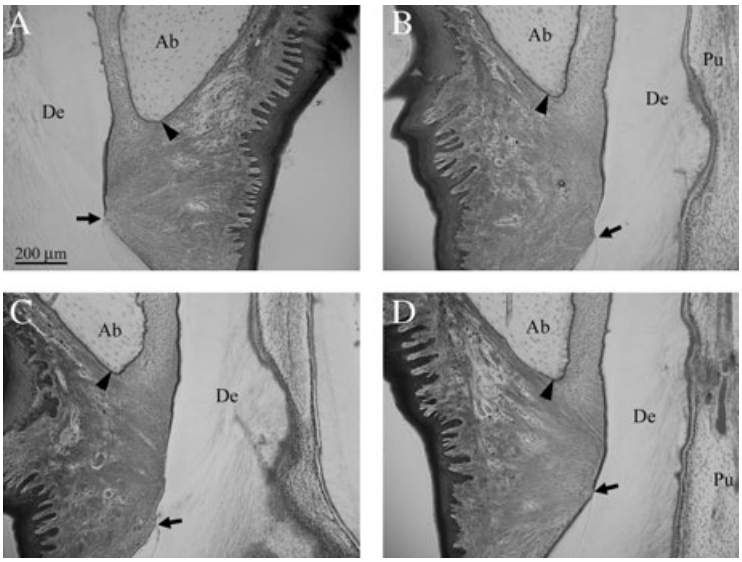

Fig. 3. Villanueva bone staining observation. Low magnification of undecalcified frontal sections in the maxillary secondary molar from rats on day 90 in the nonligatured control (A), ligatured control (B), MBP-L group (C) and MBP-H group (D). Ab, alveolar bone; De, dentin; Pu, pulp. The arrows indicate the cement–enamel junction, and the arrowheads indicate the osteoid. MBP-H group, rats receiving a high dose (1.0%) of milk basic protein in powder diet; MBP-L group, rats receiving a low dose (0.2%) of milk basic protein in powder diet.

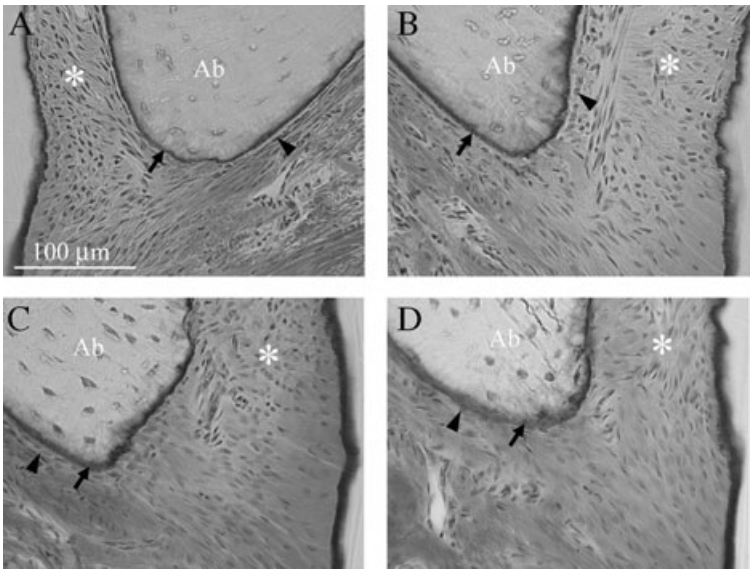

Fig. 4. High magnification of Villanueva bone staining. (A) Day 90 in the nonligatured control, (B) day 90 in the ligatured control, (C) day 90 in the MBP-L group and (D) day 90 in the MBP-H group. Ab, alveolar bone. The asterisk indicates the periodontal ligament, the arrows indicate the osteoid, and the arrowheads indicate the osteoblast. MBP-H group, rats receiving a high dose (1.0%) of milk basic protein in powder diet; MBP-L group, rats receiving a low dose (0.2%) of milk basic protein in powder diet.

MC3T3-E1 cells, that the active components of milk basic protein, related to bone formation, may comprise a high-mobility-group-like protein and a kininogen fragment 1.2 (25,26). An *in vivo* study also showed the stimulatory effects of milk basic protein on bone metabolism (4–6). Takada *et al.* (4,5) reported that supplementation with milk whey protein and milk basic protein increased

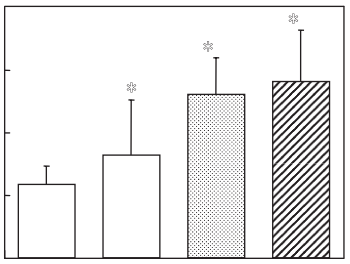

Fig. 5. Osteoid thickness of alveolar bone. Data show the mean ± standard deviation from six rats in each group. $*p < 0.05$ compared with the nonligatured control. † $p < 0.05$ compared with the ligatured control. MBP-H, rats receiving a high dose (1.0%) of milk basic protein in powder diet; MBP-L, mice receiving a low dose (0.2%) of milk basic protein in powder diet.

hydroxyproline, a collagen-related amino acid, in the rat femur, and enhanced bone strength in ovariectamized rats. These results support our histological finding that osteoid thickness was significantly increased in the low- and high-milk basic protein groups on day 90 compared with nonligatured and ligatured controls. It is possible that milk basic protein increased the activity of osteoblasts surrounding the alveolar bone and the production of bone matrix proteins, such as collagen and osteocalcin. Therefore, it is suggested that milk basic protein could be an effective dietary supplement for the recovery of alveolar bone loss.

Measurements of osteoid and mineralization on the outer cortical bone are normally used to analyze drug effects on bone formation under conditions of osteoporosis in animal models (27). It is reported that cuboidal osteoblasts, which are lined with osteoid, have a high alkaline phosphatase activity in the forming surface of cortical bone in growing rats (28). There are many reports that some agents, such as cyclooxygenase inhibitor and bisphosphonate, can reduce bone resorption of outer alveolar bone (18,27). Although fewer reports have focused on the outer area of alveolar bone, we believe that the increased osteoid thickness reflects milk basic protein-induced osteoblastic activity. In the histological observation of periodontal tissues, it is interesting

that not only alveolar bone, but also periodontal ligaments and gingival tissue, recovered in the MBP-H group. As it is unclear whether or not milk basic protein affected the connective tissues, further study is necessary to examine this finding.

Analysis using micro-CT showed the tendency to recover alveolar bone in the MBP-L group; however, the alveolar bone level in the MBP-L group was similar to that in the ligatured control on days 45 and 90. On the other hand, osteoid thickness of alveolar bone in the MBP-L group showed a significant increase compared with the ligatured controls, indicating that these results did not correlate with the alveolar bone heights of MBP-L group using micro-CT. A previous report, using dual-energy X-ray absorptiometry, showed that administration of 0.1% milk basic protein for 17 wk increased the bone mineral density of the trabecular rich region, but did not increase the bone mineral density of the cortical rich region in the femur, in ovariectomized rats (6). However, the report demonstrated that the mechanical strength of the central femur, which consists of cortical bone, was significantly increased by such a low dose of milk basic protein administration. In this study, we speculate that it takes more time for the mineralization and maturation of cortical bone to take place after the formation of osteoid or low-mineralized bone. From these findings, a longer-term experiment may be required to detect the effect of a low dose of milk basic protein on mineralization

In conclusion, we clarified the stimulatory effect of milk basic protein on alveolar bone formation after ligature-induced rat periodontitis. Our findings suggest that systemic administration of milk basic protein may be effective for the recovery of alveolar bone loss in periodontitis.

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