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Topical administration of simvastatin recovers alveolar bone loss in rats

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Background and Objective: Simvastatin, a cholesterol-lowering drug, has been reported to show anabolic effects on bone metabolism. We examined the effects of simvastatin *in vitro* using cultured rat calvaria cells and *in vivo* using periodontitis-induced rats.

Material and Methods: Alkaline phosphatase activity and bone nodule formation were measured in cultured rat calvaria cells. Nylon ligature was placed around the maxillary molars of Fischer male rats for 20 d to induce alveolar bone resorption. After ligature removal, simvastatin was topically injected into the buccal gingivae for 70 d and then microcomputed tomography and histological examinations were performed.

Results: Simvastatin maintained high alkaline phosphatase activity and increased bone nodule formation in rat calvaria cells in a dose-dependent manner, showing that simvastatin increased and maintained a high level of osteoblastic function. Microcomputed tomography images revealed that treatment with simvastatin recovered the ligature-induced alveolar bone resorption, showing a 46% reversal of bone height. Histological examination clarified that low-mineralized alveolar bone was formed in simvastatin-treated rats.

Conclusion: These findings demonstrate that simvastatin has the potential to stimulate osteoblastic function and that topical administration of simvastatin may be effective for the recovery of alveolar bone loss in rats.

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Periodontitis results in the loss of connective tissue and bone support and is a major cause of tooth loss in adults (1). For regeneration of the destroyed periodontal tissues, various clinical procedures, such as guided tissue regeneration and autogenous bone grafts, and applications of enamel matrix derivative (EMD), bone morphogenetic proteins and fibroblast growth factor, have been used. As these procedures require surgery, there are some disadvantages, such as post-operative pain and the risk of immunoreaction. It would therefore be desirable to develop other useful and safer treatments for periodontal regeneration instead of surgery.

Statins – 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA)-reductase inhibitors – are widely used for lowering serum cholesterol. It has been reported that several statins, such as simvastatin and lovastatin, have anabolic effects on bone metabolism in *in vitro* and *in vivo* studies (2). It has also been clarified that statins stimulate osteoblastic bone formation *in vitro* and *in vivo* (3). Clinical studies have shown the beneficial effect of statins on osteoporosis (4). As periodontitis is a bone-destructive disease, the application of statins may be effective for the recovery of alveolar bone. However, few reports have investigated the effect of statins on alveolar bone defects induced by periodontitis.

In this study, we first examined the *in vitro* effects of simvastatin on osteoblastic differentiation in cultured rat calvaria cells, and, second, investigated the *in vivo* effects of topical administration of simvastatin on alveolar bone loss induced in rat experimental periodontitis.

Material and methods

Rat calvaria cell culture

Rat calvaria cells were prepared from the calvariae of 21-d-old-fetal Wistar rats, as described previously (5,6). Briefly, cells isolated by sequential digestion with a collagenase mixture were cultured in α-Eagle's minimum essential medium containing 10% fetal bovine serum and antibiotics. After 24 h, rat calvaria cells were trypsinized and seeded at a density of 3300 cells/cm^2 into α-Eagle's minimum essential medium containing 10% fetal bovine serum, 50 μg/mL of ascorbic acid and 2 mM β-glycerophosphate. Different concentrations of simvastatins (1.0, 3.0 and 5.0 μM; Wako Pure Chemical Industries, Ltd, Osaka, Japan) were added to the medium and the cells were cultured for 28 d. Experiments were repeated at least three times using triplicate rat calvaria cell cultures.

Determination of alkaline phosphatase activity and bone nodule formation

Alkaline phosphatase activity and bone nodule formation were measured to determine the effect of simvastatin on osteoblastic differentiation in rat calvaria cells. To assess alkaline phosphatase activity, cells were scraped into 50 mM Tris-HCl buffer (pH 7.4), sonicated and centrifuged at 2000 *g* for 10 min. The enzyme activity in the supernatant was determined using *p*-nitrophenyl phosphate as the substrate, according to a previous report (7). To assess bone nodule formation, cells were washed in phosphate-buffered saline, fixed with 10% neutral-buffered formalin and stained by the von Kossa technique, according to a previous report (8). The area of bone nodule stained as dark dots was determined using computer software (NIH image version 1.61, Bethesda, MD, USA).

Animal procedures

Fifteen male Fischer rats (8 wk of age; Clea Japan Inc., Tokyo, Japan) 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. All animal experiments complied with the guidelines approved by the Animal Research Control Committee of Tokushima University Graduate School. 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 20 d, five rats were killed and their maxillae were collected as the baseline. These samples were defined as the control on day 0. In the remaining 10 rats, the nylon thread was removed on day 0 and the rats were divided into two groups (five rats per group): the ligatured control group; and the simvastatin-treated group. Fifty microlitres of phosphate-buffered saline, alone, or containing 0.2 mg of simvastatin, was injected into the subperiosteum at the buccal area of the maxillary second molar twice a week for 70 d. This dose was determined according to the report of Mundy *et al.* (2), who demonstrated the stimulatory effect on bone formation by injecting a similar dose of simvastatin into mouse calvariae (10 mg/kg/d). The left maxillae without ligature were used as the nonligatured control. For bone labeling, tetracycline (12 mg/kg) and calcein (10 mg/kg) were injected subcutaneously 10 and 5 d, respectively, before the rats were killed.

Microcomputed tomography analysis

Maxillae were scanned by microcomputed tomography (Hitachi Medico, Tokyo, Japan). The computed tomography was set as follows: pixel size, 1024 × 1024; slice thickness, 12 μm; magnification, 10×; voltage, 50 kV; and electrical current, 0.1 mA. Three-dimensional images were made using a computer. The distance from the buccal cemento-enamel junction to the alveolar bone crest of the second molar was measured as a marker of bone height.

Histomorphometric analysis

For preparing undecalcified sections, the maxillae on day 70 were embedded in methylmethacrylate resin. The frontal sections, parallel to the mesial root of the second molar, were prepared to ≈20 μm thicknesses. All samples were stained with Villanueva bone staining before embedding (9). All sections were observed under a light microscope and a fluorescent microscope (Microphoto V series VFD; Nikon, Tokyo, Japan) at 100× and 400× magnifications, respectively. The fluorescence line of tetracycline and calcein, and autofluorescence of Villanueva bone staining, were detected through 420–490 nm using fluorescence microscopy. The thickness of low-mineralized bone was measured by estimating the orange-colored area under fluorescence microscopy.

Statistical analysis

All values measured are expressed in the figures as the mean ± standard deviation. Significance between groups was estimated using one-way analysis of variance and Fischer's protected least significance test, and *p*-values of < 0.05 were considered significant.

Results

Alkaline phosphatase activity and bone nodule formation in rat calvaria cells

As shown in Fig. 1A, the alkaline phosphatase activity of rat calvaria cells gradually increased until day 14 in all groups. No significant difference in alkaline phosphatase activity was observed between the groups at any time point, and between the groups and the control from days 7–14, at different concentrations of simvastatin. However, on day 17, the alkaline phosphatase activity was significantly reduced in the control group, whereas a high activity was maintained in the simvastatin-treated groups.

In the experiments for the bone nodule formation assay, rat calvaria cells were cultured with or without simvastatin for 28 d. Figure 1B shows a photograph of mineralized bone nodules, visible as dark dots. When the bone nodule area was measured, its formation was significantly increased by simvastatin treatment in a

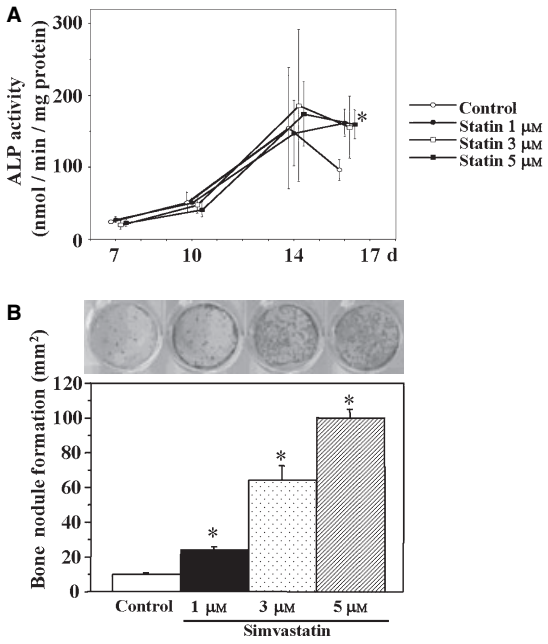

Fig. 1. Simvastatin increases alkaline phosphatase activity and bone nodule formation in cultured rat calvaria cells. Time course of alkaline phosphatase activity (A), photograph of bone nodules stained by von Kossa on day 28 and the measurement of the bone nodule area (B). Data show the mean ± standard deviation for triplicate samples in three separate experiments. $*p < 0.05$ compared with the control group. ALP, alkaline phosphatase.

dose-dependent manner, showing a ninefold increase at 5 μM simvastatin (Fig. 1B).

Microcomputed tomography analysis of alveolar bone

In Fig. 2A(i and ii), three-dimensional images from microcomputed tomography revealed a clear decrease in the buccal alveolar bone of the second molar in the ligatured control group compared with the nonligatured control group on day 0. Interestingly, exposure of the buccal furcation area induced by ligaturing for 20 d was evident, as shown in Fig. 2A(ii). Although similar profiles of alveolar bone continued until day 70 in the ligatured control group (Fig. 2A, iii and iv), it was clear that ligature-induced alveolar bone loss was partially reversed in the simvastatin-treated group on day 70 (Fig. 2A, v). In addition, a rough bone surface was observed at the buccal bone surface in the simvastatin-treated group. When the distance from the cemento-enamel junction to the alveolar bone crest was measured, it was found to be longer in the ligatured control group than in the nonligatured control on day 0 (281 ± 33 vs. 757 ± 97 μm, respectively) (Fig. 2B). On day 70, this distance was significantly shorter in the simvastatin-treated group compared with the ligatured control (491 ± 72 and 691 ± 101 μm, respectively). The distance from the cemento-enamel junction to the alveolar bone crest of the nonligatured control on day 70 (256 ± 29 μm) was similar to that on day 0 (281 ± 33 μm). From calculating these bone heights (256, 691 and 491 μm), 46% of the total bone loss was found to be reversed in the simvastatin-treated group.

Histomorphometric analysis of alveolar bone

The low magnification of Villanueva bone staining on day 0 revealed the destruction of periodontal tissues, showing a decreased thickness of the epithelial layer, irregular collagen fibers and a rough surface of alveolar bone beneath the nylon thread in the ligatured control group (Fig 3A,B). In addition, the alveolar bone crest had regressed from the cemento-enamel junction as a result of the ligature procedure. On day 70, such disturbance of periodontal tissues was not observed in nonligatured and ligatured control groups (Fig. 3C,D). On the other hand, the amount of alveolar bone had increased in the simvastatin-treated group (Fig. 3E arrowheads), confirming the results obtained from microcomputed tomography analysis. However, bone was added predominantly more at the horizontal site than at the vertical site. The newly formed bone structure in the simvastatin-treated group showed a rough surface at the buccal site and it was stained yellow, which is known to indicate a low-mineralized area.

In highly magnified sections, destruction of bone tissues was observed in the ligatured control group but not in the nonligatured control group on day 0 (Fig. 4A,B). In addition to inflammatory cells (lymphocytes and monocytes), multinuclear osteoclast-like cells were detected and a resorptional concave of alveolar bone was observed in the ligatured control group on day 0 (Fig. 4B, arrows). On day 70, the surface of the alveolar bone became smooth in the ligatured control, and osteoblasts were in alignment on the bone surface, showing an image similar to that of the nonligatured controls (Fig. 4C,D). In the simvastatin-treated group, the newly formed rough bone was stained yellow and osteocytes were included in the low-mineralized area (Fig. 4E, asterisks). Inflammatory cells were not observed around the newly formed bone in the simvastatin-treated group.

Under the fluorescent microscope, mature mineralized bone was observed as a dark-green-colored mass and low-mineralized bone was shown as a bright red-colored mass including osteocytes, and osteoid was observed as dark red-colored lines (Fig. 5A,B). The amount of low-mineralized bone (bright red-colored area) increased at the buccal site and alveolar bone crest in the simvastatin-treated group. Such bone additions were not observed in the ligatured control group. The fluorescent labelings of tetracycline

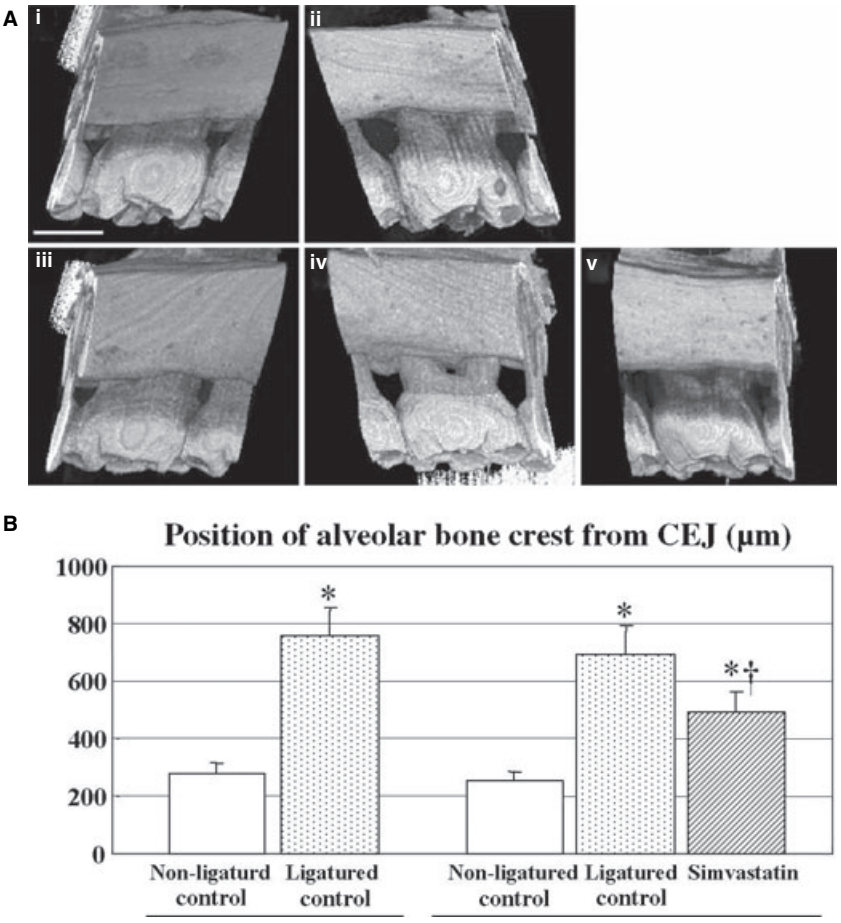

Fig. 2. (A) Microcomputed tomography images show the recovery of alveolar bone loss by simvastatin treatment. The frontal section of the maxillary secondary molar of rats on day 0 [the nonligatured control group (i), and ligatured control group (ii)] and on day 70 [the nonligatured control group (iii), the ligatured control group (iv) and the simvastatin-treated group (v)] is shown. (B) Alveolar bone height was recovered in the simvastatin-treated group. The distance from the cemento-enamel junction to the alveolar bone crest was measured by microcomputed tomography. Data show the mean ± standard deviation from five rats per group. Bar, 1.0 mm. $*p < 0.05$ compared with the nonligatured control group. $\dagger p < 0.05$ compared with the ligatured control group. CEJ, cemento-enamel junction.

(yellow) and calcein (bright green) were clearly detected outside the alveolar bone in the ligatured control group. However, these labeling lines were not detected in the area of low-mineralized bone in the simvastatin-treated group. When the bone volume was estimated, the low-mineralized bone thickness was greater in the simvastatin-treated group than in the ligatured control (Fig. 5C).

Discussion

It is possible, as shown by the *in vitro* findings of this study, that simvastatin may affect the function of rat calvaria cells because alkaline phosphatase activity continued at a higher level in the simvastatin-treated group than in the control group, and more bone nodules were formed in the simvastatin-treated group. The anabolic effect of simvastatin has been demonstrated in various osteoblastic cells: MG63 (2), MC3T3-E1 (3) and rat bone marrow cells (3). These reports showed that simvastatin could increase osteoblastic markers, such as alkaline phosphatase activity, mineralized bone nodule formation and bone matrix protein expression. It can be speculated from these *in vitro* studies that the administration of simvastatin constantly stimulates osteoblastic functions.

Experimental animal models have been used to clarify the pathogenesis of periodontal diseases and to develop new periodontal therapy. Ligature methods have been accepted as a useful experimental model of periodontitis with alveolar bone resorption (10–13). In this study, periodontal tissues, including alveolar bone, epithelial cells and connective tissues, were damaged by ligature procedures, although inflammatory cells and osteoclasts disappeared from the periodontal tissues after 20 d of ligature. Using experimentally ligatured rats, Lima *et al.* (12) and Bezerra *et al.* (13) showed that the inflammatory change peaked from 7 to 11 d after periodontitis. It is thought that, as the inflammatory reactions did not continue for a long time in the ligatured rats, osteoclasts and inflammatory cells disappeared beneath the nylon thread after 20 d of ligature. In addition, the experimental bone loss persisted for 70 d after removing the ligature in this study. In our previous study, this bone loss persisted for 45–90 d, even after removal of the ligature (14). These data indicate that this model is useful for evaluating the change of alveolar bone after the formation of the bone defect. Amstad-Jossi & Schroeder (15) reported that the distance between the cemento-enamel junction and the alveolar bone crest increased in an age-dependent manner at the palatal and lingual side of the upper and lower second molars in rats. In the present study, we could not clearly detect the age-related changes in the controls. It is thought that the increase of the distance between the cemento-enamel junction and the alveolar bone crest was caused by the ligature procedures and the influence of age-related change was minimal in this experimental model.

Topical administration of bisphosphonates to periodontal tissues has demonstrated that bisphosphonates prevent alveolar bone loss and decrease the number of osteoclasts in rat experimental periodontitis (16,17). Topical injection of bisphosphonates was found to prevent tooth movement

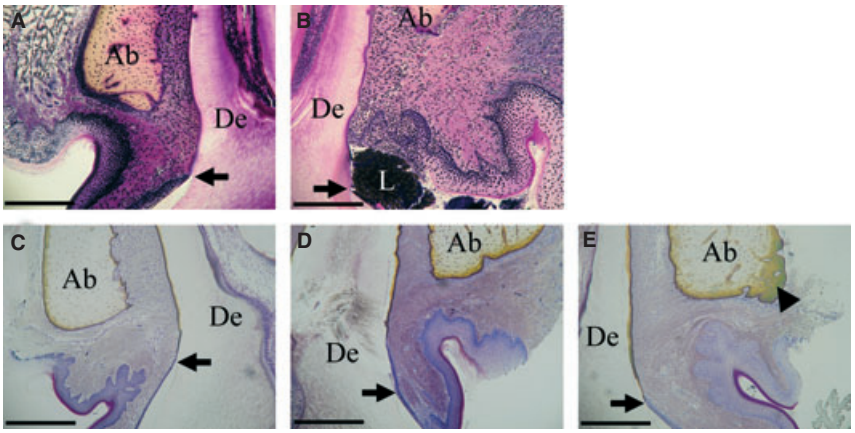

Fig. 3. Villanueva bone-staining observation. Low magnification of undecalcified frontal sections in the maxillary secondary molar from rats in the nonligatured control group (A) and ligatured control group (B) on day 0, and from rats in the nonligatured control group (C), ligatured control group (D) and simvastatin-treated group (E) on day 70. Bar, 250 μm. Ab, alveolar bone; De, dentin; L, ligature. The arrows indicate the cemento-enamel junction, and the arrowhead indicates newly formed bone.

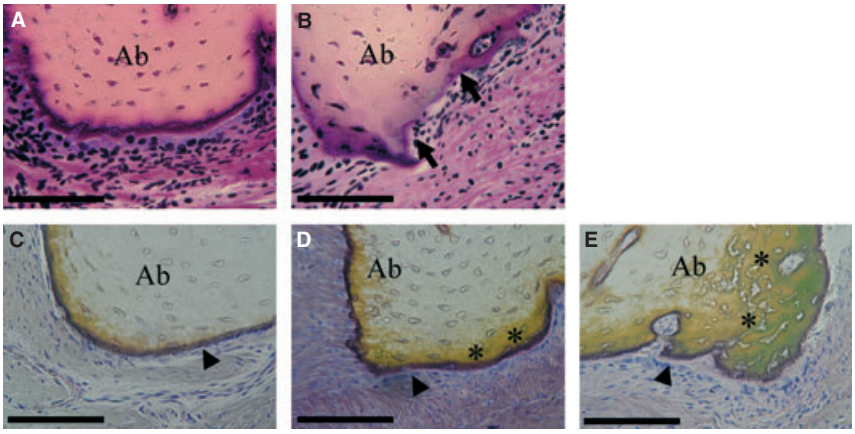

Fig. 4. Villanueva bone-staining observation. High magnification of undecalcified frontal sections in the maxillary secondary molar from rats in the nonligatured control group (A) and ligatured control group (B) on day 0, and from rats in the nonligatured control group (C), ligatured control group (D) and simvastatin-treated group (E) on day 70. Bar, 100 μm. Ab, alveolar bone; asterisk, low-mineralized bone. The arrows indicate resorptional concave, and the arrowhead indicates osteoblasts.

and root resorption after providing orthodontic force and inhibited relapse of the tooth after removal of the orthodontic appliance in rats (18,19). Topical administration is generally beneficial for the treatment of periodontitis because it requires less agent and results in fewer side-effects compared with systemic administration. However, further investigations are necessary before topical administration of bisphosphonate can be performed in periodontitis patients because repeated local injections is not a suitable method of delivery. A slow-release device may be suitable, but this has yet to be developed.

Stein *et al.* (20) reported an inflammatory reaction in rat soft tissues and mandibular bone where a high dose of simvastatin was locally applied. They indicated that the inflammatory reaction was decreased by reducing the simvastatin dose from 2.2 mg/30 μL to 0.5 mg/30 μL. In the present study, topical administration of simvastatin (0.2 mg/50 μL) significantly increased the volume of alveolar bone without any inflammatory reactions being observed in periodontal tissues. These findings indicate that the topical administration of a low dose of simvastatin is effective for recovering periodontal tissues involving alveolar bone destruction. It has been reported that new cortical bone formation increased when simvastatin was locally applied into the periosteum of mouse calvariae and rat mandibular bone (2,20,21). It was also demonstrated that the anabolic effect via the systemic administration of simvastatin to ovariectomized rats was greater in cortical bone than in trabecular bone (22). These reports strongly support our findings that the topical administration of simvastatin increases the outer layer of alveolar bone. It has been reported that simvastatin increases bone formation and also inhibits bone resorption (23). We

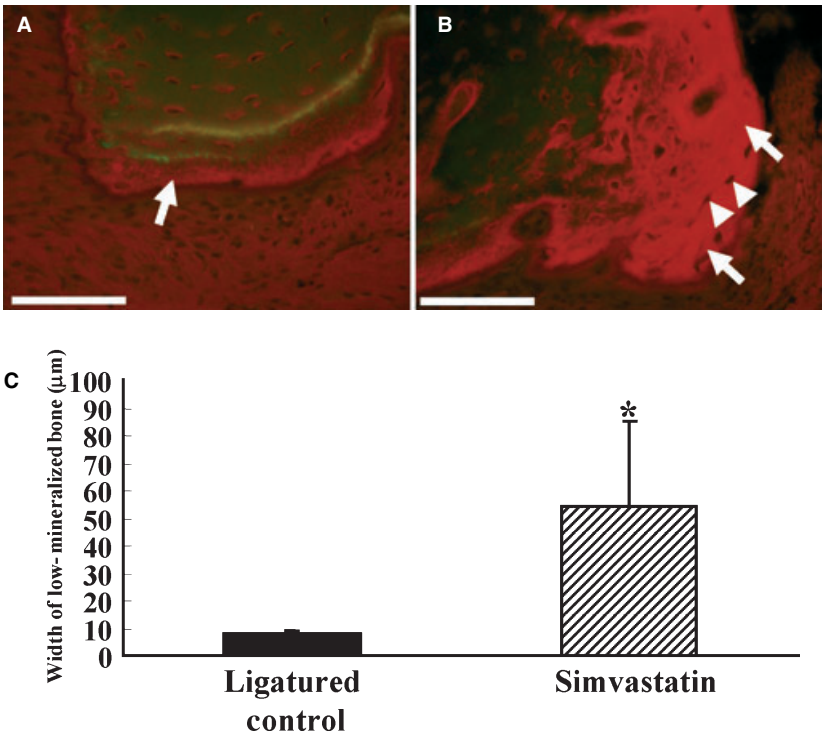

Fig. 5. Fluorescence microscopy of undecalcified frontal sections from rats of the ligatured control group (A) and simvastatin-treated group (B) on day 70. Arrows indicate low-mineralized bone, and arrowheads indicate osteocytes. Bar, 100 μm. (C) Low-mineralized bone thickness increased in rats of the simvastatin-treated group. Data show the mean ± standard deviation of five rats per group. $*p < 0.05$ compared with the ligatured control group.

speculate that the present findings only show the stimulatory effect on bone formation because simvastatin was locally applied after bone resorption was induced by the ligature procedures and then further resorption did not take place in the controls. The labeling lines of tetracycline and calcein were not detected in the low-mineralized bone area in this study. In general, these fluorescent lines are known to be clearly visible in mature bone (24). Our results may indicate that newly formed alveolar bone is still immature and the rough surface is one phenotype showing such immaturity. When these immature osteoids become mature bone, the fluorescent lines may appear. Previous studies have shown that low-mineralized bone is formed as a result of high bone turnover (9,25). Our histological analyses showed that many osteocytes were incorporated into the low-mineralized bone in the simvastatin-treated rats. Recently, the function of osteocytes in bone metabolism has been discussed, in which nucleobindin, a calcium-binding proteins, modulates bone matrix maturation by localizing in osteocytes and osteoblasts (26). It has also been reported that sclerostin is secreted from osteocytes and inhibits the mineral maturation of compact bone (27). Thus, as osteocytes have an important role in maturation of the bone matrix, low-mineralized bone induced by simvastatin may have the potency to form mature alveolar bone.

From the histological findings, it is interesting that not only alveolar bone, but also periodontal ligament and connective tissue, recovered in the simvastatin-treated group. However, the effect of simvastatin on cementum formation was not clear in this study. It has been reported that simvastatin enhanced the proliferation and osteoblastic differentiation of human periodontal ligament cells via inhibition of the mevalonate pathway (28). As it is unclear whether or not simvastatin affects connective tissues *in vivo*, further study is necessary to examine this point.

In conclusion, we clarified the stimulatory effect of simvastatin on alveolar bone formation after ligature-induced periodontitis in rats. Our findings suggest that the topical administration of simvastatin may be effective for the recovery of alveolar bone loss in periodontitis.

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