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# The role of macrophages in the periodontal regeneration using Emdogain® gel

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*Background and Objective:* Emdogain® gel is clinically used as a periodontal regenerative material. However, the mechanism of the regeneration has not been completely elucidated. Although many studies have focused on the regenerative effect of Emdogain on connective tissue attachment and alveolar bone, the role of macrophages and the expression of growth factors remains unclear in the regeneration stimulated by Emdogain gel *in vivo*. The aim of this study was to investigate the effect of Emdogain gel on the expression of cytokines and growth factors by macrophages *in vivo* using a newly devised rat experimental periodontitis model.

*Material and Methods:* Rat experimental periodontitis was induced by elevating a full-thickness gingival flap and ligating silk threads around the first molars of the mandible. At 14 d after inducing experimental periodontitis, Emdogain gel or propylene glycol alginate was applied to the furcation area. The rats were killed 7 and 14 d after treatment with propylene glycol alginate or Emdogain gel. The expression of cytokines and growth factors, and the regeneration of periodontal tissue, were examined by histochemical and immunohistochemical methods.

*Results:* Fourteen days after the induction of periodontitis, the resorption of alveolar bone at furcation was observed and cytokines such as interleukin-1β, transforming growth factor-β1, receptor activator of nuclear factor-κB ligand, receptor activator of nuclear factor-κB and osteoprotegerin were found. In the Emdogain-treatment group, the formation of new acellular cementum and, more remarkably, recovery of the bone, were observed. The new bone formation ratio in the Emdogain treatment group was significantly higher than that of the propylene glycol alginate treatment group. Although the expression of cytokines such as interleukin-1β, transforming growth factor-β1, receptor activator of nuclear factor-κB ligand and receptor activator of nuclear factor-κB was very low, bone morphogenetic protein-2- and bone morphogenetic protein-4-expressing macrophages were observed close to the root, and bone morphogenetic protein-4-expressing macrophages were mainly observed close to the bone surface at the furcation in the Emdogain-treatment group.

*Conclusion:* These results suggest that wound-healing macrophages may express bone morphogenetic protein and play an important role in the regeneration of periodontal tissue at the furcation following the application of Emdogain gel.

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Alveolar bone loss is one of the symptoms of periodontitis. Although the regulation of periodontal tissue regeneration is still under investigation, much effort has been spent investigating the promotion of periodontal wound healing. Osteoblasts and osteoclasts are responsible for balancing bone formation and bone resorption (1,2). Osteoclasts cause resorption of the alveolar bone in periodontitis. It has been reported that receptor activator of nuclear factor-κB ligand (RANKL) is essential for the induction of osteoclastogenesis (3). Osteoblasts and bone marrow stromal cells produce RANKL and its signal is transduced by the specific receptor, receptor activator of nuclear factor-κB (RANK), which is expressed on the cell surface of osteoclast progenitors (4). Osteoprotegerin is produced by osteoblasts and inhibits osteoclastogenesis by being involved in the competitive binding of RANK and RANKL (5). It was also reported that interleukin-1β produced by macrophages affects osteoclastogenesis indirectly via osteoblast RANKL expression and directly via osteoclast maturation (6,7). Takayanagi *et al.* reported that synovial fibroblasts and T cells also express RANKL on the cell membrane, and T cells directly release soluble RANKL and activate osteoclast formation (8,9). These results suggest that inflammatory cells also play an important role in bone resorption.

Bone morphogenetic protein was reported by Urist as a noncollagenous protein that is present in demineralized bone and induces bone formation (10). Bone morphogenetic protein is also known to play important roles in periodontal tissues. Several studies suggest that bone morphogenetic proteins modulate osteogenesis, fibrillogenesis and cementogenesis in periodontal tissue (11–13).

Macrophages participate in both repair and destruction in the process of wound healing (14,15). It has been reported that macrophage cell lines produce osteoinductive signals, including bone morphogenetic protein-2 (16). In an experiment using mouse osteoarthritis models, synovial lining macrophages expressed several growth factors, such as transforming growth factor-β, bone morphogenetic protein-2 and bone morphogenetic protein-4, and played an important role in osteocyte formation (17). In an immunohistochemical study that investigated the human wound process of the bone fracture, macrophages expressing growth factors were reported to be associated with osteoinduction (18). Therefore, growth factors produced by macrophages are associated with wound healing and bone formation. Champagne *et al.* suggested that the roles of macrophages in osseous wound healing were deeply affected by conditions where macrophages were present. Macrophages are able to convert to wound-healing macrophages that produce growth factors or to inflammatory macrophages that produce inflammatory cytokines (16). This suggests that during the wound-healing process of the periodontium, the switch from inflammatory macrophages to wound-healing macrophages may be important.

Recently, periodontal regenerative therapy using Emdogain® gel has been investigated clinically and the usefulness of regenerative therapy with Emdogain gel for mandibular class II furcation lesions has been suggested (19,20). Emdogain gel is considered to activate the signaling pathway for secreting growth factors in wounds (21). Emdogain gel stimulates the proliferation of periodontal ligament cells and enhances alkaline phosphatase activity and the secretion of transforming growth factor-β1 and insulin-like growth factor 1 (22). Although there are many reports on the regeneration of connective tissue attachment and the effect of Emdogain gel on periodontal ligament, few studies have focused particularly on the behavior of macrophages and the expression of growth factors in the regeneration of periodontal tissues as stimulated by Emdogain gel *in vivo*.

The aim of the present study was to investigate the effect of Emdogain gel on the role of inflammatory cells, including macrophages, and on the expression of cytokines and growth factors, such as interleukin-1β, transforming growth factor-β1, RANKL, RANK, osteoprotegerin, bone morphogenetic protein-2 and bone morphogenetic protein-4, in a newly devised experimental periodontitis model by using immunohistochemical and histochemical methods.

## Material and methods

### Experimental design

Twenty, 10-wk-old male Sprague-Dawley rats (body weight 320–360 g) were used. All experimental procedures were conducted in accordance with the animal experimental guidelines of Kyushu University. Under general anesthesia (intramuscular injection of sodium pentobarbitone), the reflection of full-thickness mucoperiosteal flaps were made at the right and left first molars of the mandibula. Periodontal ligament and cementum were mechanically removed. Then, silk thread (Φ 0.1–0.15 mm), soaked for 1 d in Brain–Heart Infusion broth containing 5 mg/mL of yeast extract, was ligated around the cervix of the first molars, as shown in Fig. 1A. The animals were randomly divided, 14 d after the induction of experimental period ontitis, into the following three groups: the nontreatment group ($n = 4$); the Emdogain group ($n = 8$); and the propylene glycol alginate group ($n = 8$). In the nontreatment group, rats were immediately killed. In the Emdogain and propylene glycol alginate groups, the silk thread was removed and the mucoperiosteal flaps were reflected again. Then, either Emdogain or propylene glycol alginate was applied to the furcation area of the first molars. For root surface treatment, 24% EDTA was used, and sterilized isotonic sodium chloride solution was used for washing. The wound was left to heal for 7 and 14 d. At the end of the experiments, the animals were killed and mandibular blocks were taken for histological examination.

### Histological preparation

Each animal was fixed by an intracardiac perfusion of periodate–lysine–paraformaldehyde fixative (3% paraformaldehyde, 0.01 M $NaIO_4$, 0.075 M lysine in 0.025 M phosphate

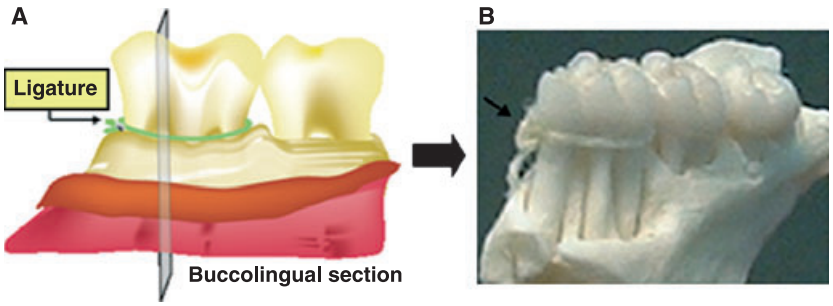


*Fig. 1.* Schema of the surgical procedure (A) and typical bone destruction (B). (A) After the reflection of full-thickness mucoperiosteal flaps were made at the right and left first molars of the mandibula, the periodontal ligament and cementum were mechanically removed. Then, silk thread (Φ 0.1–0.15 mm), soaked for 1 d in Brain–Heart infusion broth containing 5 mg/mL of yeast extract, was ligated around the cervix of the first molars. (B) Alveolar bone destruction in the first mandibular molar 14 d after the induction of experimental periodontitis. Significant bone resorption in the first molar was observed as compared with the second and third molars. The arrow shows the ligature in the mandibular first molar.

buffer, pH 7.3). The mandibular blocks were dissected, including first molars, and soaked in the same fixative at 4°C for 12 h. After fixation, the mandibular blocks were demineralized with 10% EDTA in a solution of 7.5% polyvinylpyrrolidone (Sigma Chemical Co., St Louis, MO, USA) at 4°C for 2 mo. The decalcified specimens were washed, in turn, with phosphate-buffered saline containing 10% sucrose, for 6 h, with phosphate-buffered saline containing 15% sucrose for 6 h, and with phosphate-buffered saline containing 20% sucrose for 12 h at 4°C. Tissue blocks were freeze-embedded in OCT compound (Milles Scientific, Naperville, IL, USA). Frozen specimens were serially step-sectioned (5 μm) parallel to the long axis of the tooth in a bucco–lingual plane. After the first section cut from each tissue was stained with hematoxylin and eosin for routine histological assessment, sections with good morphology were included in the study.

### Histochemical and immunohistochemical analysis

Tartrate-resistant acid phosphatase (TRAP) staining was performed with naphthol AS-BI phosphate (Sigma) as the substrate and with hexazonium pararosaniline (Sigma) as the coupler, in a solution containing 100 mM L(+) sodium tartrate. Nonspecific alkaline phosphatase staining was performed with naphthol AS-BI phosphate as the substrate and Fast Red violet LB salt (Sigma) as the coupler. After staining, each section was counterstained with methyl green (Sigma).

Immunohistochemical examination was performed using the enzyme-marker-polymer method. In brief, endogenous peroxidases were inactivated by immersing the sections in 0.3% hydrogen peroxide for 30 min. After washing with phosphate-buffered saline, sections were incubated with normal goat serum or rabbit serum for 30 min to block nonspecific binding. The sections were subsequently incubated for 12 h at 4°C with the primary antibodies shown in Table 1. When using mouse monoclonal antibodies or rabbit polyclonal antibodies as second antibodies, immunohistochemical staining was performed with Histofine Simple Stain rat MAX-PO(MULTI) (Nichirei Co., Tokyo, Japan). When using goat polyclonal antibodies as second antibodies, the sections were incubated with biotinylated antigoat IgG (Nichirei) for 10 min at room temperature. After washing with phosphate-buffered saline, the sections were incubated with peroxidase-labeled streptavidin (Nichirei) for 5 min at room temperature. After washing with phosphate-buffered saline, the chromogenic reaction was performed with 3,3'-diaminobenzidine (DAB) solution (Nichirei). Finally, all sections were counterstained with methyl green.

In addition, two-color staining with anti-ED1 and anti-bone morphogenetic protein-4 were performed to demonstrate the existence of ED-1/bone morphogenetic protein-4-positive macrophages. The sections were incubated first with anti-bone morphogenetic protein-4 and subsequent steps were performed to develop the

*Table 1.* Antibodies used for immunohistochemistry in this study

| Antibody name | Specificity | Type of antibodies | Supplier |
|---|---|---|---|
| Anti-rat CD68 (ED1) | Rat macrophage, weakly peripheral blood granulocytes | Mouse monoclonal antibody | Serotec |
| Anti-rat CD5 | Rat thymocytes, T cells, B-cell subset | Mouse monoclonal antibody | Biolegend |
| Anti-IL-1β | Rat IL-1β | Rabbit polyclonal antibody | Endogen |
| Anti-TGF-β1 | Rat, human TGF-β1 | Rabbit polyclonal antibody | Promega |
| Anti-RANKL | Mouse, rat, human RANKL | Rabbit polyclonal antibody | Santa Cruz |
| Anti-RANK | Mouse, rat, human RANK | Goat polyclonal antibody | Santa Cruz |
| Anti-OPG | Mouse, rat, human OPG | Goat polyclonal antibody | Santa Cruz |
| Anti-BMP-2 | Mouse, rat, human BMP-2 | Goat polyclonal antibody | Santa Cruz |
| Anti-BMP-4 | Mouse, rat, human BMP-4 | Goat polyclonal antibody | Santa Cruz |
| Anti-keratin | Wide range of human keratin subunits | Rabbit polyclonal antibody | Nichirei |
| Anti-PCNA | Mainly synthesis period | Mouse monoclonal antibody | Zymed |

BMP, bone morphogenetic protein; IL, interleukin; OPG, osteoprotegerin; PCNA, proliferating cell nuclear antigen; RANK, receptor activator of nuclear factor-κB; RANKL, receptor activator of nuclear factor-κB ligand; TGF, transforming growth factor.

visualization using DAB solution, as described above. After washing with phosphate-buffered saline, anti-ED-1 was placed on the sections and incubated. Immunoreactivity was visualized using the Histofine Simple Stain MAX-AP (M) (Nichirei). Tetramisole hydrochloride (Sigma) was used to block endogenous alkaline phosphatase activity. Finally, the sections were counterstained with methyl green.

### Quantitative analysis of the number of antibody-positive cells

Antibody-positive cells close to the root surface and bone surface at the furcation were counted using an ocular grid ($7 \times 7$ mm$^2$) at 200× magnification of the light microscope, as shown in Fig. 2. Measurement from 10 sections for each was averaged.

### Measurement of the height of periodontal ligament and new bone formation ratio

The height of the periodontal ligament, extending from the cementum to the bone surface, and the amount of new bone formation, were measured using an ocular grid ($7 \times 7$ mm$^2$) and measurement software (NIH IMAGE), as shown in Fig. 2. The new bone formation ratio (%) was calculated as follows:

New bone formation =
[(the height of the new bone/
the height of the bone defect) × 100].

### Statistical analysis

The differences between the Emdogain and propylene glycol alginate groups were analyzed by the Student's *t*-test. Values were considered to be significantly different when the *p*-value was $< 0.05$ or $< 0.01$.

## Results

### Histological findings: nontreatment group

Fourteen days after the induction of experimental periodontitis, the resorption of alveolar bone at the furcation was advanced, as shown in Fig. 1B. In histological analyses, TRAP-positive osteoclasts were observed on the bone surface, and many macrophages and T cells were present around the ligature and the abscess and were adjacent to the bone surface. Interleukin-1β-expressing macrophages and transforming growth factor-β1-expressing macrophages were observed near the abscess. RANKL and RANK were expressed in the vicinity of the bone surface, but the expression of bone morphogenetic protein-2 and bone morphogenetic protein-4 was extremely low (Figs 3A,B and 4A–H).

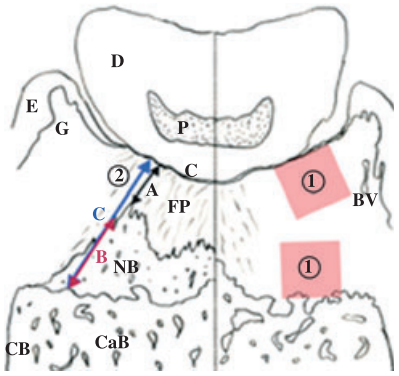


*Fig. 2.* Schema of histological analysis. ① The ocular grid ($7 \times 7$ mm) was placed close to the root dentin and the bone surface for counting the antibody-positive cells. On the dentin side, the ocular grid was placed to fit the notch points that were made around the cemento–enamel junction. On the bone side, the ocular grid was placed to fit the standard point which is the border of the cortical bone and cancellous bone. ② The height of periodontal ligament extending from the cementum to the bone surface (A) and the height of new bone (B) were measured. The new bone formation ratio was calculated as follows: new bone formation ratio (%) = [the height of new bone (B)/the height of bone defect (C)] × 100. The standard points were set at the border of the cortical bone and cancellous bone, and the four segments that are located at regular intervals from the standard point were measured. BV, blood vessel; C, cementum; CaB, cancellous bone; CB, cortical bone; D, dentin; E, epithelium; FP, furcation periodontium; G, gingiva; N, new bone; P, pulp.

### Histological findings: propylene glycol alginate treatment group

*Seven days after treatment* — The pocket epithelium extended from the cemento–enamel junction towards the central direction of the furcation. Infiltrations of macrophages and T cells were found in the gingival connective tissue below the bottom of the pocket. Neither the addition of new cementum on the root surface nor the arrangement of cementoblasts adjacent to the root surface was observed. Infiltrations of a small number of macrophages and a few T cells were found in the connective tissue near the bone surface. On the bone surface, a few, small round-shaped osteoclasts were observed and alkaline phosphatase-positive flat osteoblasts were found, but only a small number of proliferating cell nuclear antigen-positive cells were scattered. Interleukin-1β, transforming growth factor-β1, RANKL, RANK and osteoprotegerin were not observed near the bottom of the pocket or the bone surface. The number of bone morphogenetic protein-2- or bone morphogenetic protein-4-positive cells adjacent to the bone surface was extremely low (Figs 3C and 5A–E).

*Fourteen days after treatment* — The epithelium of the pocket had moved more deeply towards the centre of the furcation. Neither the formation of the cementum nor cementoblasts strongly positive for alkaline phosphatase were observed on the root surface. Although a small number of macrophages and T cells were found near the bone surface, the number of macrophages expressing bone morphogenetic protein-2 or bone morphogenetic protein-4 were very low. The expression of interleukin-1β, transforming growth factor-β1, RANKL, RANK and osteoprotegerin was extremely low. Osteoclasts were not found on the bone surface, but alkaline phosphatase positive-flat osteoblasts were detected. In addition, a small number of proliferating cell nuclear antigen-positive cells were scattered (Figs 3D and 6A–D).

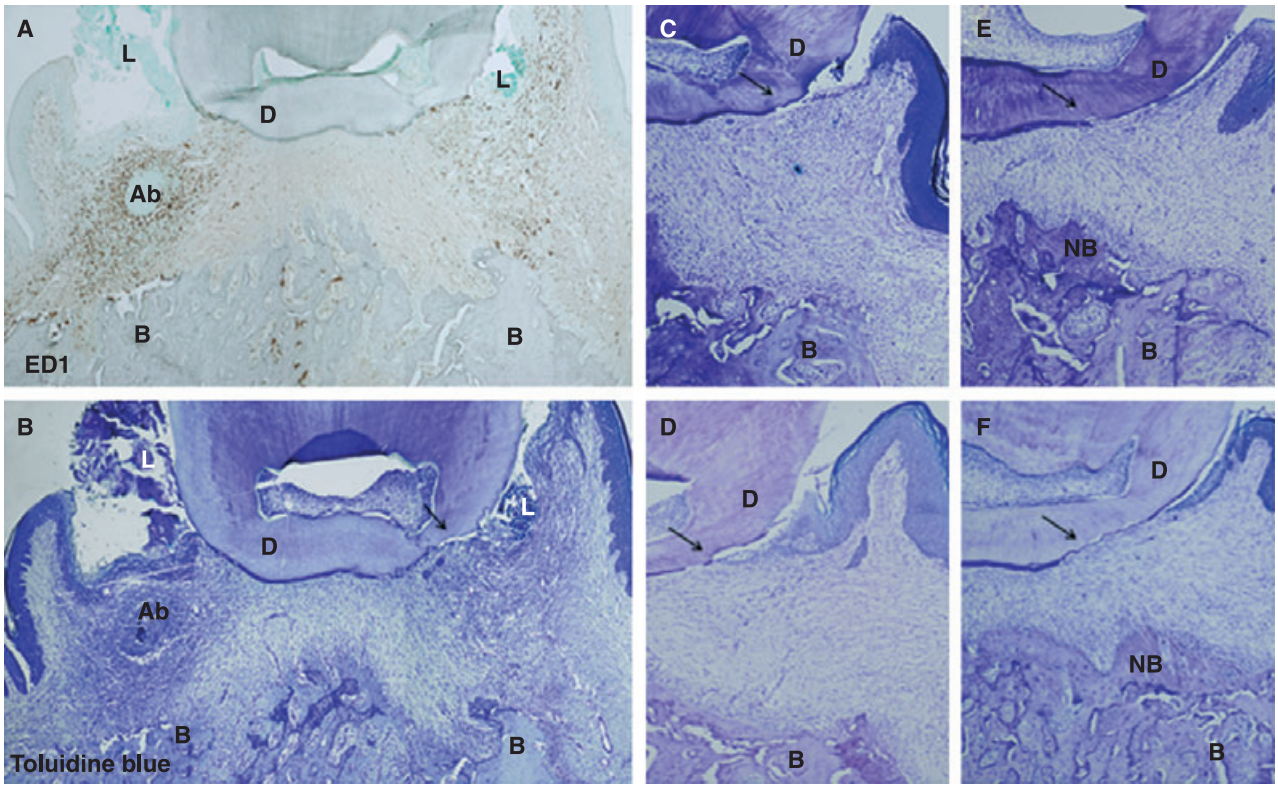


*Fig. 3.* (A) Macrophages immunostained with ED1 antibody in the nontreatment group. (B) Toluidine blue staining in the nontreatment group. (C) Toluidine blue staining in the propylene glycol alginate group 7 d after treatment. (D) Toluidine blue staining in the propylene glycol alginate group 14 d after treatment. (E) Toluidine blue staining in the Emdogain group 7 d after treatment. (F) Toluidine blue staining in the Emdogain group 14 d after treatment. Magnification ×40. The arrow shows the notch. Ab, abscess; Bo, bone; De, dentin; L, ligature; NB, new bone.

### Histological findings: Emdogain gel treatment group

*Seven days after treatment* — Although the addition of thin new acellular cementum was found on the root surface, cementoblasts showing strong alkaline phosphatase activity were present at very low levels in the new acellular cementum. However, thick collagen fibers were arranged in a radial manner from new cementum towards the bone surface, and alkaline phosphatase-positive osteoblasts with a rectangular or an oval shape were polylayer-arranged on the bone surface. In addition, the formation of new bone, which was distinguished clearly from the existing bone, was observed. The fibroblasts extending from new cementum showed alkaline phosphatase activity, and a small number of bone morphogenetic protein-2- and a large number of bone morphogenetic protein-4-expressing macrophages were observed in the vicinity of fibroblasts. The expression of interleukin-1β, RANKL and RANK was very low, whereas transforming growth factor β1 and osteoprotegerin were detected in a few cells. On the other hand, a small number of macrophages were observed in the connective tissue near the bone surface, but infiltration of T cells was low and the expression of interleukin-1β, transforming growth factor-β1, RANKL, RANK and osteoprotegerin was weak. In addition, few osteoclasts were found on the bone surface and osteoblasts expressing bone morphogenetic protein-2 and bone morphogenetic protein-4 were detected. Although a large number of orbicular and polygonal macrophages expressing bone morphogenetic protein-4 were observed in the vicinity of bone surface, few macrophages expressing bone morphogenetic protein-2 were found (Figs 3E and 5F–J).

*Fourteen days after treatment* — In the vicinity of the acellular new cementum, alkaline phosphatase-positive cells, which seemed to be cementoblasts, were observed. Thick collagen fibers were arranged in a radial manner from new cementum to the bone surface. The volume of new bone formation was much greater than that 7 d after treatment with Emdogain gel. Rectanglar or oval alkaline phosphatase-positive osteoblasts were observed on the new bone surface. A small number of macrophages expressing bone morphogenetic protein-2 and bone morphogenetic protein-4 were found apart from the new cementum. The expression of interleukin-1β, transforming growth factor-β1, RANKL, RANK and osteoprotegerin was weak, both at the bottom of the pocket and in the vicinity of the bone surface. Although a small number of macrophages were observed in the connective tissue close to the bone

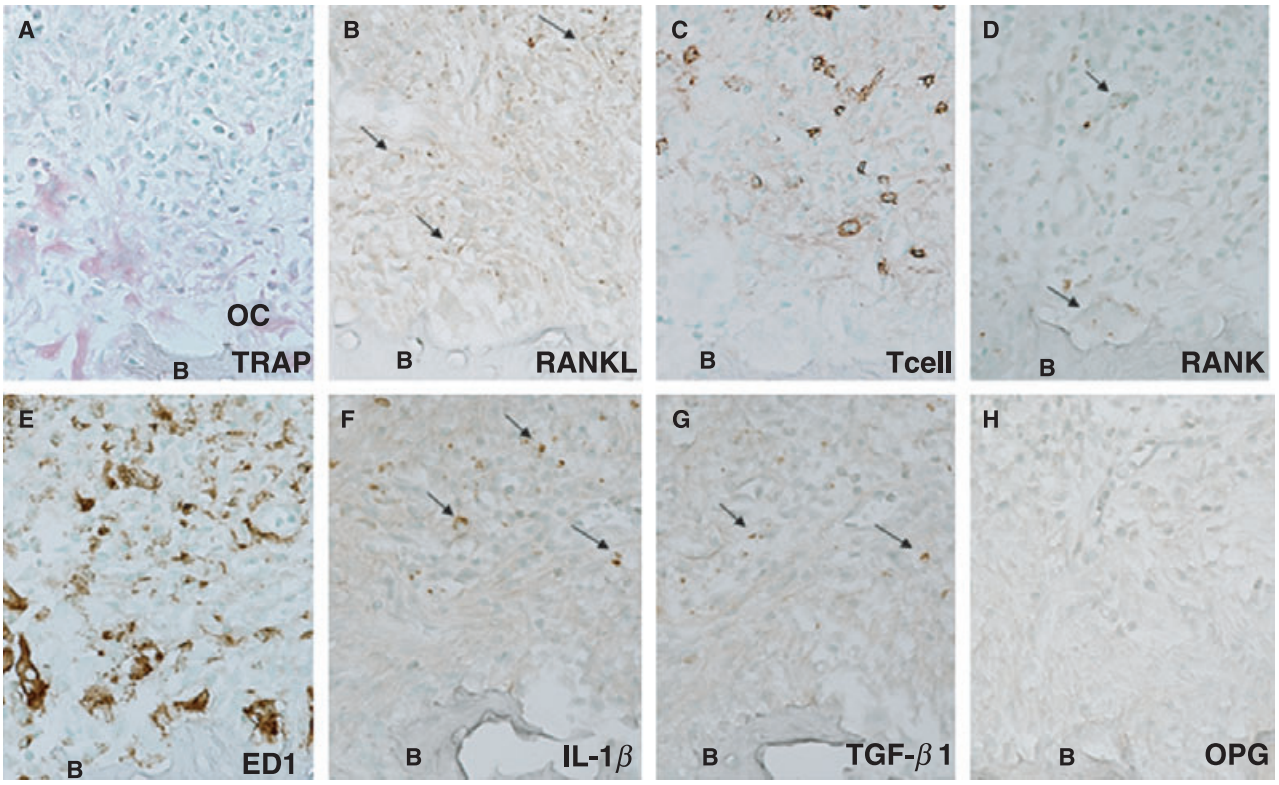


*Fig. 4*. Tartrate-resistant acid phosphate staining (A) and immunohistochemical staining for receptor activator of nuclear factor-κB ligand (B), T cells (C), receptor activator of nuclear factor-κB (D), macrophages (E), interleukin-1β (F), transforming growth factor-β1 (G) and osteoprotegerin (H) in the nontreatment group. Magnification ×400. Bo, bone; Oc, osteoclast. IL-1β, interleukin-1β; OPG, osteoprotegerin; RANK, receptor activator of nuclear factor-κB; RANKL, receptor activator of nuclear factor-κB ligand; TGF-β1, transforming growth factor-β1; TRAP, tartrate-resistant acid phosphatase.

surface, very few osteoclasts were found on the bone surface, and osteoblasts expressing bone morphogenetic protein-2 and bone morphogenetic protein-4 were present. Moreover orbicular or polygonal macrophages expressing bone morphogenetic protein-4 were observed in the vicinity of the bone surface. However, the expression of bone morphogenetic protein-2 was very weak (Figs 3F and 6E–H).

Two-color immunohistochemical staining was performed to demonstrate the existence of ED-1/bone morphogenetic protein-4-positive macrophages. As shown in Fig. 7, some ED-1-positive macrophages expressed bone morphogenetic protein-4.

### Measurement of the height of periodontal ligament and new bone formation ratio

In the Emdogain group at 7 and 14 d, the height of the periodontal ligament was significantly lower than in the propylene glycol alginate group. The new bone formation ratio of the Emdogain group was significantly higher than that of the propylene glycol alginate group, suggesting more active bone formation in the Emdogain group than in the propylene glycol alginate group throughout the experimental period (Fig. 8A,B).

### Quantitative analysis of antibody-positive cells

The number of ED1-positive cells close to the root surface and the bone surface at both 7 and 14 d after treatment with Emdogain gel and propylene glycol alginate was significantly lower than in the nontreatment group 14 d after the induction of experimental periodontitis. The number of ED1-positive cells close to the root surface at both 7 and 14 d after treatment with Emdogain gel was significantly lower than in the propylene glycol alginate treatment group. The number of ED1-positive macrophages 14 d after treatment with Emdogain was significantly lower than at 7 d after treatment. Similar results were obtained for the area close to the bone surface (Fig. 9A,B).

The number of CD5-positive cells close to the root surface and bone surface at both 7 and 14 d after treatment with Emdogain and propylene glycol alginate was also significantly lower compared with those present 14 d after induction of experimental periodontitis. At 14 d after treatment, the number of CD5-positive cells of the propylene glycol alginate group that were close to the root surface was significantly higher compared with the Emdogain group (Fig. 9C,D).

The number of osteoclasts showed a significant decrease after treatment, being observed only at very low levels on the alveolar bone surface in both treatment groups (Fig. 9E).

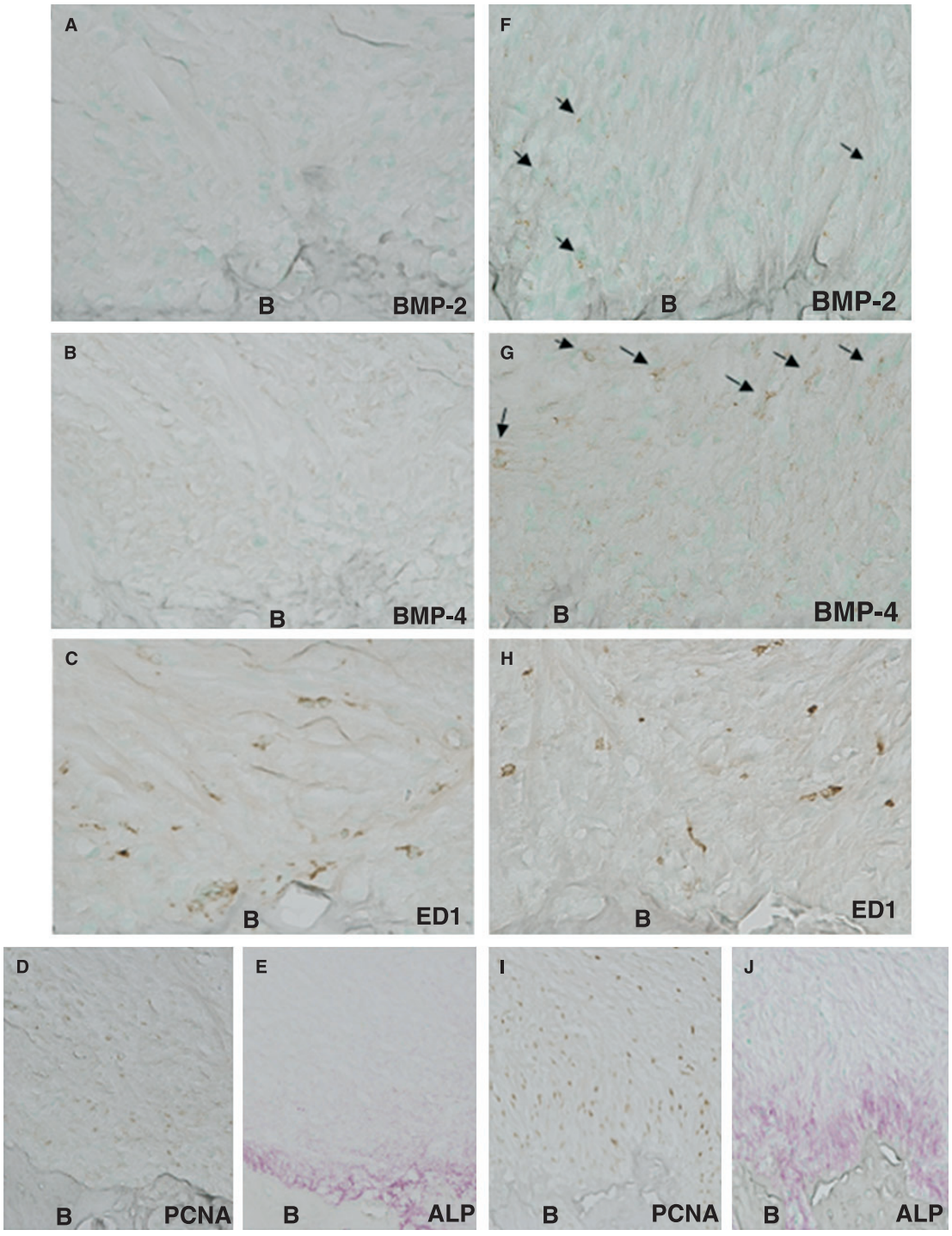


*Fig. 5.* Immunohistochemical staining for bone morphogenetic protein-2 (A,F), bone morphogenetic protein-4 (B,G), macrophages (C,H) and proliferating cell nuclear antigen (D,I), and alkaline phosphatase staining (E,J), 7 d after treatment with propylene glycol alginate (A–E) or Emdogain gel (F–J). Magnification: ×400 (A–C,F–H) or ×200 (D,E,I,J). The arrow shows bone morphogenetic protein-2-positive cells, which seemed osteoblasts (F). The arrow shows bone morphogenetic protein-4-positive cells, which seemed macrophages (G). Bo, bone. ALP, alkaline phosphatase; BMP, bone morphogenetic protein; PCNA, proliferating cell nuclear antigen.

The number of interleukin-1β-positive cells and transforming growth factor-β1-positive cells were significantly lower in both treatment groups than in the nontreatment group 14 d after the induction of experimental periodontitis. There was no significant difference between Emdogain and propylene glycol alginate groups in the

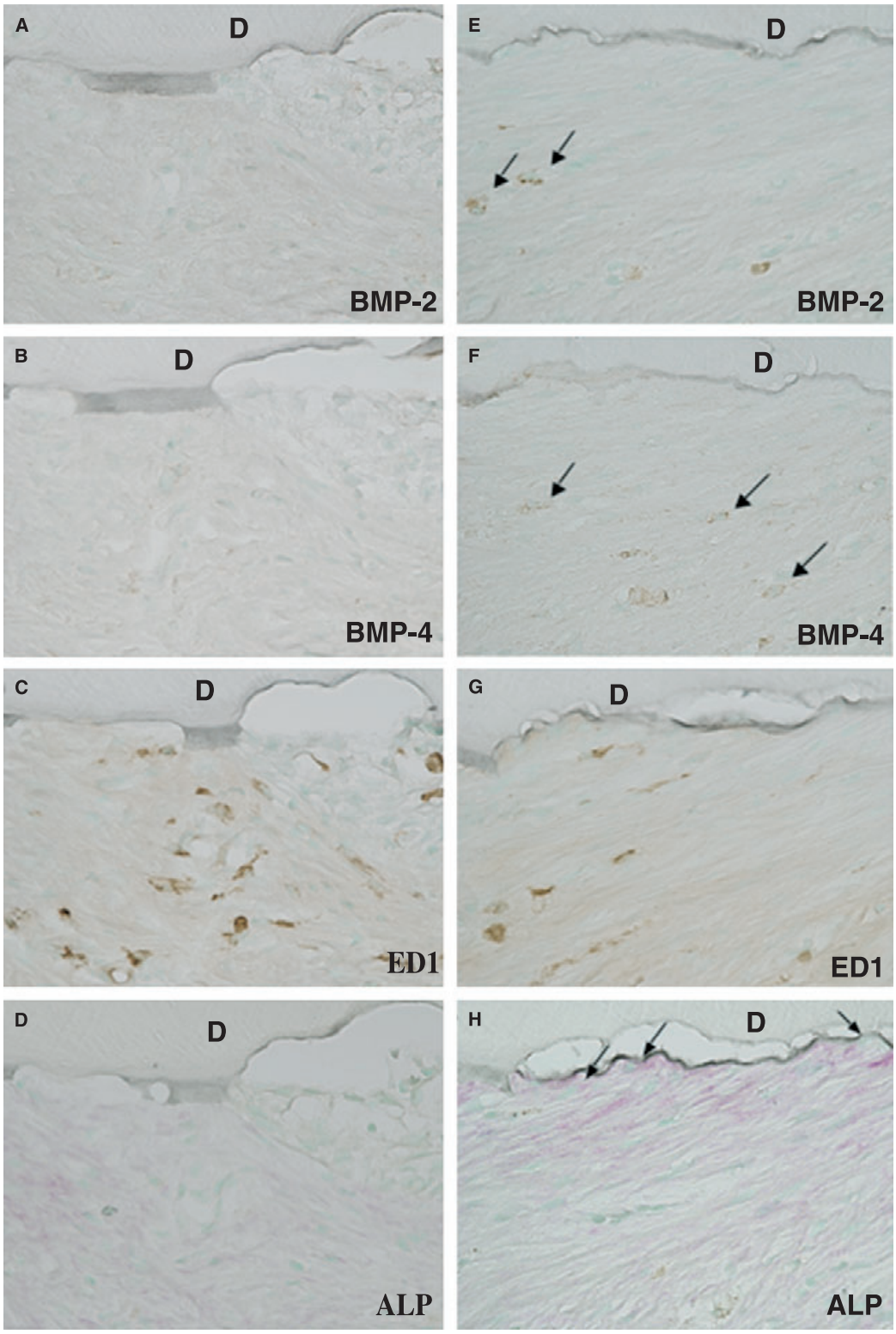


*Fig. 6.* Immunohistochemical staining for bone morphogenetic protein-2 (A,E), bone morphogenetic protein-4 (B,F) and macrophages (C,G), and alkaline phosphatase staining (D,H), 14 d after treatment with propylene glycol alginate (A–D) or Emdogain (E–H). Magnification ×400. Although ED1-positive cells were observed, very few bone morphogenetic protein-2- or bone morphogenetic protein-4-positive cells were observed in the propylene glycol alginate-treatment group. Bone morphogenetic protein-2- or bone morphogenetic protein-4-positive cells, which stained with ED1, were found in the Emdogain-treatment group. Cementoblast-like cells (arrow) were observed in the vicinity of the new acellular cementum (H). De, dentin. ALP, alkaline phosphatase; BMP, bone morphogenetic protein.

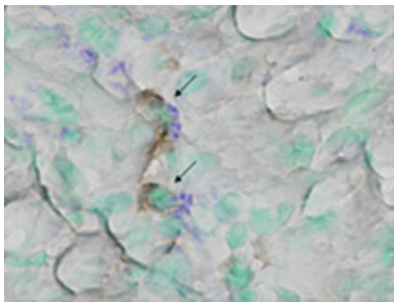

*Fig. 7.* Double staining with anti-ED1 and anti-bone morphogenetic protein-4. The blue color and the brown color show ED1-positive cells and bone morphogenetic protein-4-positive cells, respectively. The arrows show ED-1/bone morphogenetic protein-4-positive cells.

area close to the root surface and the bone surface (Fig. 10A–D).

The number of RANKL-positive cells was also significantly lower in both treatment groups compared with the nontreatment group 14 d after the induction of experimental periodontitis. Fourteen days after treatment, the number of RANKL-positive cells close to the dentin side in the Emdogain group was significantly lower than in the propylene glycol alginate group (Fig. 10E,F).

Although few bone morphogenetic protein-2-positive cells were present in both treatment groups during the experimental period, the number of bone morphogenetic protein-2-positive cells in the Emdogain group was significantly greater than in the propylene glycol alginate group. On the other hand, the number of bone morphogenetic protein-4-positive cells close to the root surface significantly increased in both treatment groups 7 d after treatment, but the increase was greater in the Emdogain group. In addition, a significantly higher number of bone morphogenetic protein-4-positive cells were found close to the bone surface in the Emdogain group than in the propylene glycol alginate group throughout the experimental period (Fig. 11A–D).

## Discussion

Rat ligature models have been used as an experimental periodontitis model (23). In a recent study where nylon ligature was placed for 5–20 d around the cervix of maxillary second molars to induce experimental periodontitis in rats, the authors reported that osteoclasts were found on the alveolar bone crest 5 d after treatment and that osteoclasts and inflammation had disappeared 10 d after treatment in spite of the alveolar bone resorption. Furthermore, they also reported that the duration of inflammation was short and the tissue repair occurred rapidly (24). In our preliminary experiment to produce a rat ligature periodontitis model, in which silk thread only was ligated around the cervix of the teeth, neither remarkable infiltration of inflammatory cells nor appearance of osteoclasts were found on the bone surface. A significant change of the bone level was not confirmed even at 14 and 28 d. Therefore, in order to maintain the duration of inflammation and bone resorption, full-thickness flaps were reflected and silk thread, soaked in Brain–Heart Infusion broth, was tightly ligated around the cervix on the alveolar crest to promote the accumulation of plaque and delay the wound healing of the flap in the present experiment. Consequently, bone resorption continued until 14 d after the induction of experimental periodontitis and the bone defect form of the furcation was similar to that of the class II furcation involvement (Lindhe's classification), as shown in Fig. 1B. Therefore, it was suggested that this experimental periodontitis model might be suitable for investigating the effect of Emdogain. Because rapid bone resorption and the appearance of many osteoclasts and a large number of cells expressing interleukin-1β and RANKL were observed at 7 d and maximum bone resorption was observed at 14 d after inducing periodontitis, Emdogain or propylene glycol alginate treatment was carried out at 14 d in this study.

Champagne *et al.* suggested that the role of macrophages in osseous wound healing depends on environmental conditions (16). They speculated that under pro-inflammatory conditions, macrophages (inflammatory macrophages) produce tumor necrosis factor-α and lose their ability to synthesize bone morphogenetic

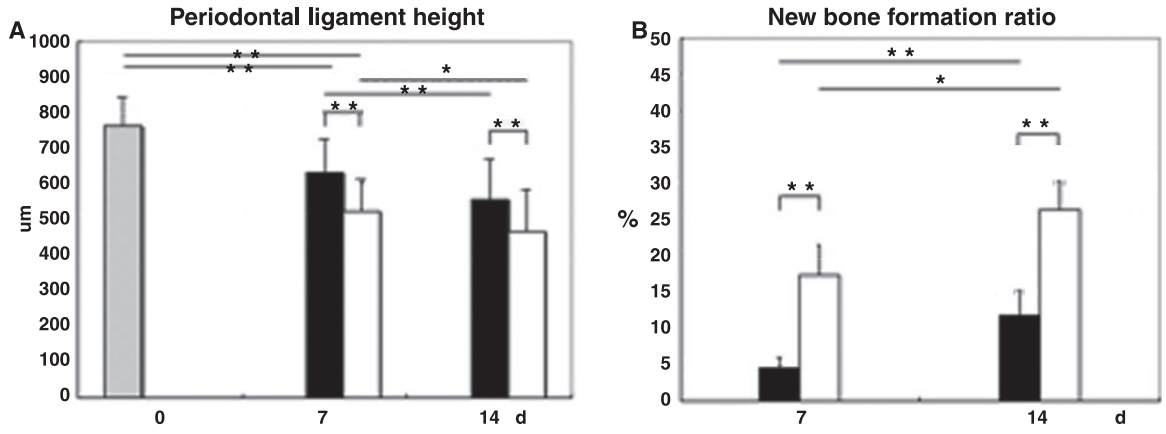


*Fig. 8.* The periodontal ligament height (A) and new bone formation ratio (B). Grey bar, nontreatment group; black bar, propylene glycol alginate group; white bar, Emdogain group. **, $p < 0.01$; *, $p < 0.05$.

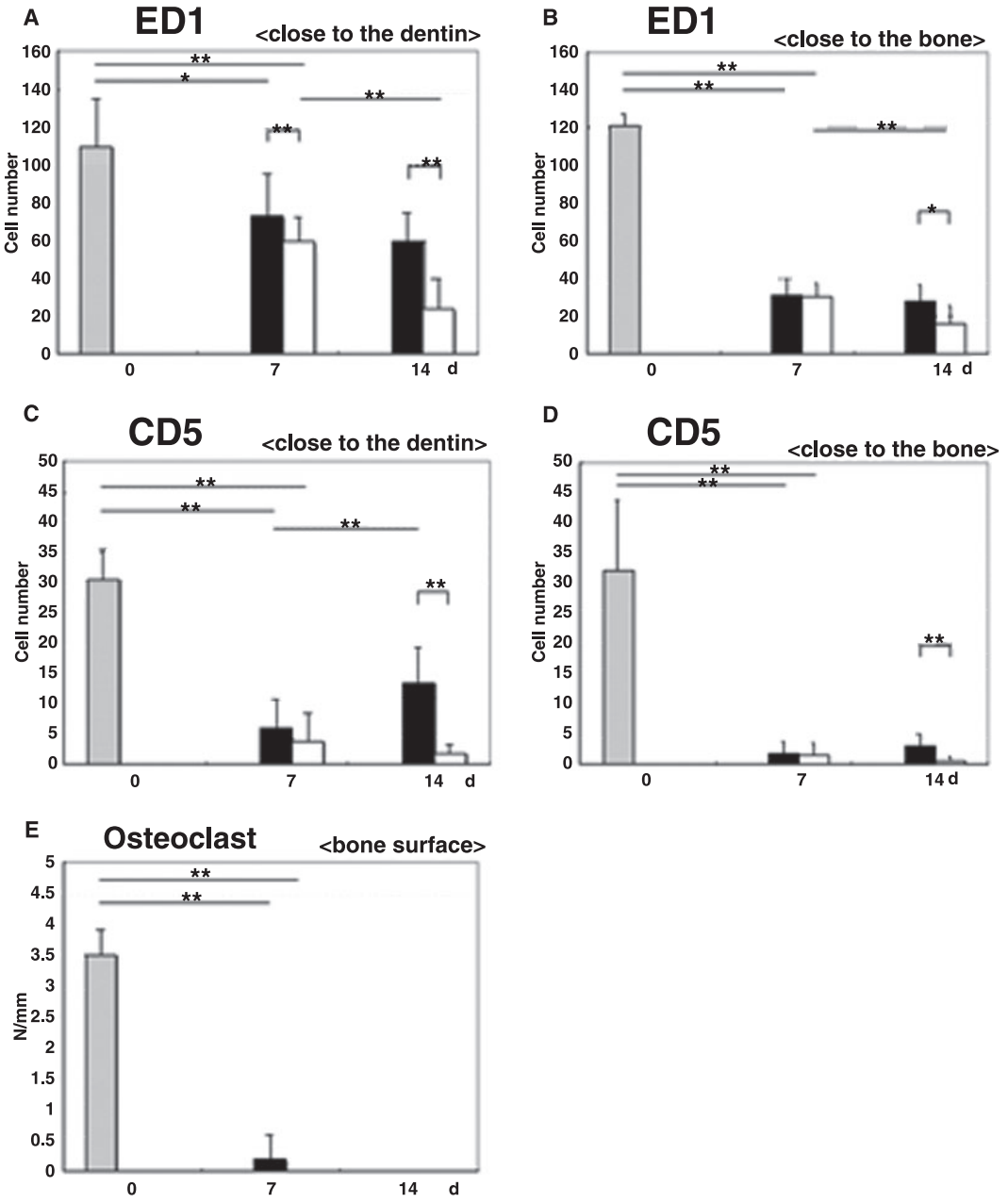


*Fig. 9.* Quantitative analysis of the number of ED1-positive cells (A,B), the number of CD5-positive cells (C,D) and the number of osteoclasts (E). Grey bar, nontreatment group; black bar, propylene glycol alginate group; white bar, Emdogain group. **, $p < 0.01$; *, $p < 0.05$.

protein-2, and, in a suitable environment for wound healing, macrophages (wound-healing macrophages) produce pro-osteogenic factors, including bone morphogenetic protein, to promote bone formation.

A recent study showed that Emdogain had anti-inflammatory effects, which attenuated the production of tumor necrosis factor-α and interleukin-8 by monocytes (25). This suggests that Emdogain may affect inflammatory macrophages and promote the conversion of inflammatory macrophages to wound-healing macrophages. Consequently, the expression of bone morphogenetic protein-2 and bone morphogenetic protein-4 by macrophages might promote regeneration of the periodontal tissue. We need more research to investigate whether Emdogain triggers the conversion of inflammatory macrophages to wound-healing macrophages. *In vitro* experiments are needed to confirm whether Emdogain can make macrophages expressing inflammatory cytokines promote the expression of bone morphogenetic protein-2 and bone morphogenetic protein-4 and the change of the intracellular signal.

In the present study, macrophages that expressed bone morphogenetic protein-4 and bone morphogenetic protein-2 were found in the Emdogain treatment group, although the expres-

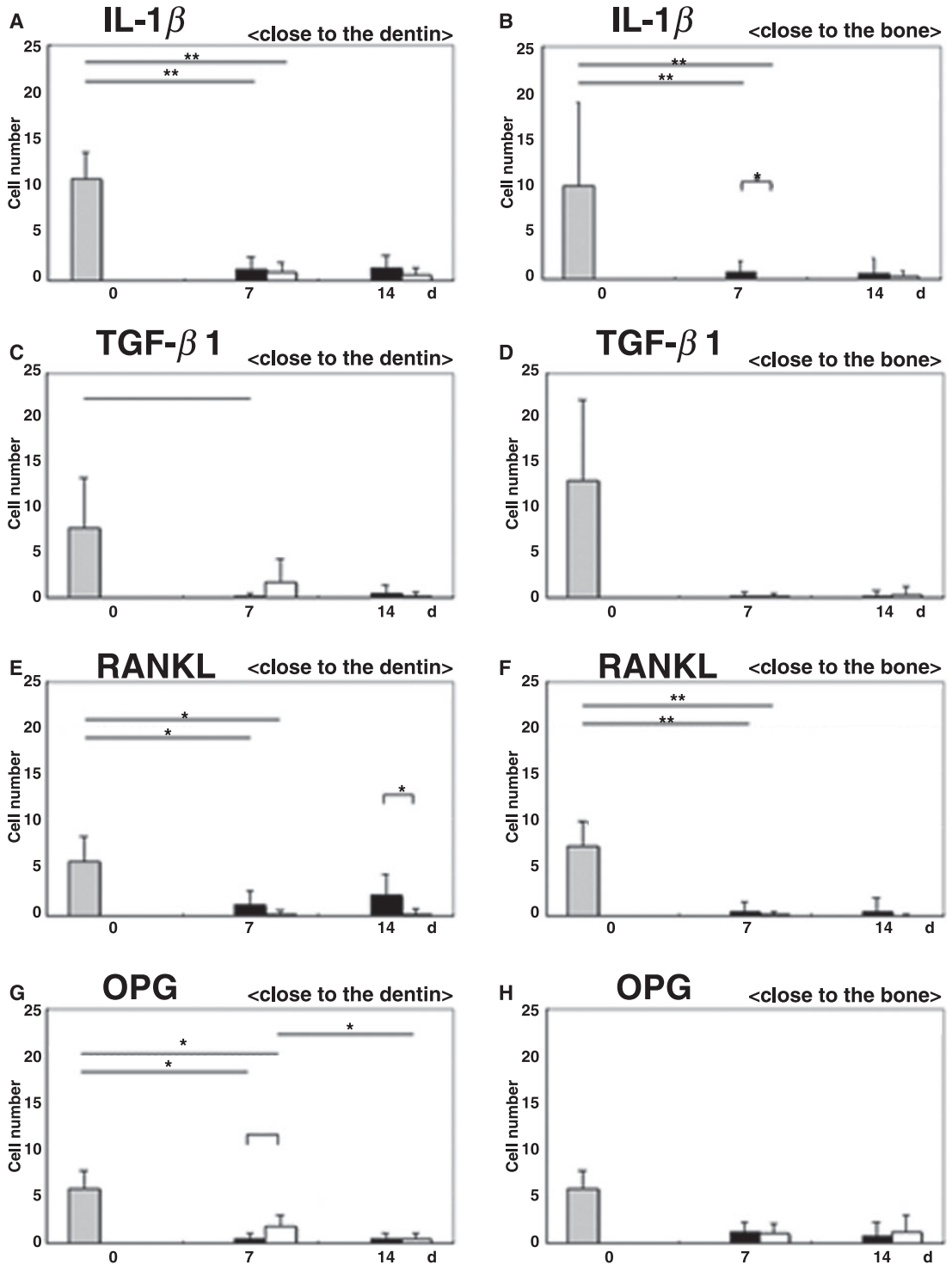


*Fig. 10.* Quantitative analysis of the number of interleukin-1β-positive cells (A,B), the number of transforming growth factor-β1-positive cells (C,D), the number of RANKL-positive cells (E,F) and the number of osteoprotegerin-positive cells (G,H). Grey bar, nontreatment group; black bar, propylene glycol alginate group; white bar, Emdogain group. IL-1β, interleukin-1β; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor-κB ligand; TGF-β1, transforming growth factor-β1. **, $p < 0.01$; *, $p < 0.05$.

sion of bone morphogenetic protein-4 was significantly higher than that of bone morphogenetic protein-2. Kawai *et al.* reported that the simultaneous transfer of bone morphogenetic protein-2 and bone morphogenetic protein-7 by electroporation into rat calf muscles induced rapid bone formation and increased the expression of endogenous bone morphogenetic protein-4. This suggests that the induction of bone morphogenetic protein-4 is related to

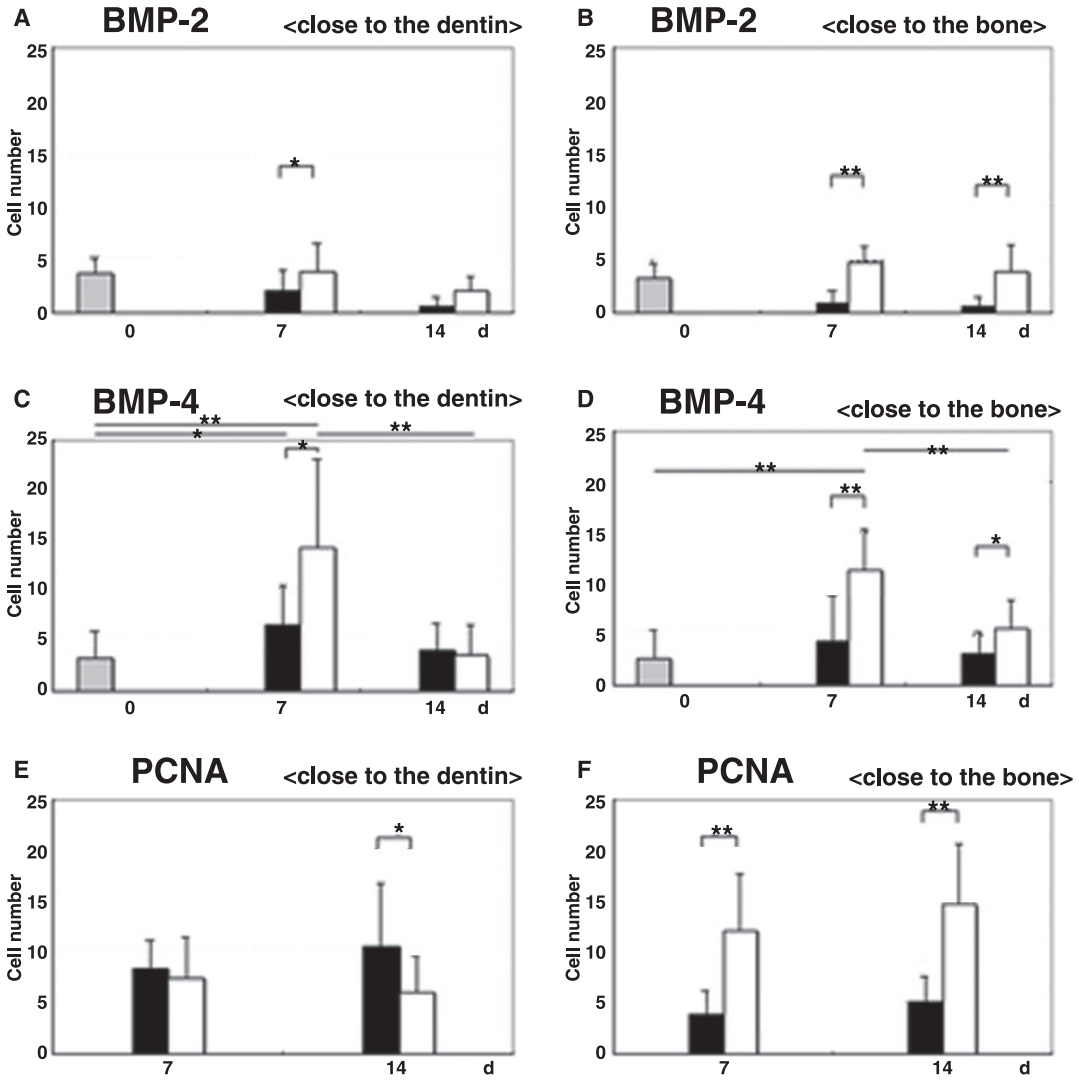


*Fig. 11.* Quantitative analysis of the number of bone morphogenetic protein-2-positive cells (A,B), the number of bone morphogenetic protein-4-positive cells (C,D) and the number of proliferating cell nuclear antigen-positive cells (E,F). Grey bar, nontreatment group; black bar, propylene glycol alginate group; white bar, Emdogain group. BMP, bone morphogenetic protein; PCNA, proliferating cell nuclear antigen. **, $p < 0.01$; *, $p < 0.05$.

rapid ossification (26). There is a possibility that the expression of bone morphogenetic protein-2 and bone morphogenetic protein-4 by macrophages might participate in the regenerative mechanism of the periodontal tissue, and the expression of bone morphogenetic protein-2 and other bone morphogenetic proteins might have some association with the expression of bone morphogenetic protein-4. More study about the mutual effect of these osteogenesis factors is required.

It has been reported that macrophages expressing transforming growth factor-β1 participated in the wound healing of skin (27). In the present study, transforming growth factor-β1 was found to be expressed near the dentin of the root surface 7 d after treatment with Emdogain. Suzuki *et al.* reported that transforming growth factor-β1-like growth factors in Emdogain gel contribute to the induction of mineralization during periodontal regeneration (28). Therefore, the production of transforming growth factor-β by macrophages might not be required because Emdogain shows a transforming growth factor-β1-like effect. Otherwise, the macrophages expressing transforming growth factor-β1 might appear at an early stage during wound healing.

Although cementoblast-like cells were not found, thin acellular cementum was found on the root surface 7 d after Emdogain treatment. This suggested that Emdogain or bone morphogenetic protein expression of macrophages might induce cementum-like material from cells, such as fibroblasts, at an early stage. Subsequently, the addition of cementum might be promoted by the differentiation from mesenchymal cells to cementoblasts.

In our preliminary experiment, active bone resorption disappeared 28 d after

inducing experimental periodontitis, but the infiltration of inflammatory cells continued and the expression of many types of cytokines (interleukin-1β, transforming growth factor-β1, RANKL, osteoprotegerin, bone morphogenetic protein-2 and bone morphogenetic protein-4) were observed (data not shown). On the other hand, 14 d after Emdogain treatment, the regeneration of periodontal tissues, including cementum formation, proliferation of collagen fibers and new bone formation, occurred. Although the infiltration of inflammatory cells significantly decreased in the vicinity of the bone surface and the expression of interleukin-1β and transforming growth factor-β1 was not found, bone morphogenetic protein-2- and bone morphogenetic protein-4-expressing macrophages were observed on the dentin. Furthermore, in the Emdogain group at 14 d, the expression of bone morphogenetic protein-2 and bone morphogenetic protein-4 was observed with polylayer-arranged osteoblasts. Macrophages mainly expressing bone morphogenetic protein-4 were observed near the osteoblasts throughout experimental period. These results suggest that inflammatory macrophages changed to wound-healing macrophages at the inflammatory site after the inflammatory stimuli were removed, and these wound-healing macrophages might play an important role for the regeneration at the furcation following the application of Emdogain® gel.

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