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The effect of Emdogain® on ectopic bone formation in tubes of rat demineralized dentin matrix

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Background: Emdogain® (EMD) is made from enamel matrix proteins (EMPs) from the tooth germ of swine and propylene glycol alginate (PGA) as a matrix. The function of EMD is known to differentiate cells of the dental follicle into cementoblasts. However, little is known about the effect of EMD on mesenchymal cells in other tissue.

Objective: The purpose of this study was to investigate whether EMD has the ability to induce hard tissue when applied with or without demineralized dentin matrix.

Methods: Half of the dentin tubes prepared from rat incisors were demineralized by treatment with 0.6 N hydrochloric acid for 3 h. EMD or PGA was injected into the demineralized or non-demineralized dentin tubes, which were then transplanted into rectus abdominis muscles. Untreated dentin tubes were also transplanted as a control. Animals were killed at 7, 14 and 21 days after the implantation.

Results: Non-demineralized dentin tubes with or without EMD or PGA did not form any hard tissue. In the demineralized group, chondrogenesis in the PGA groups occurred earlier than in the EMD groups. The expression of vascular endothelial growth factor (VEGF) mRNA in the demineralized group with PGA at day 14 was the highest. The expression of osteopontin and osteocalcin mRNAs was higher in all groups at 21 days compared with 7 or 14 days.

Conclusion: These results suggest that neither EMD nor PGA has the ability to induce hard tissue and that EMPs contained within EMD might aggregate on the dentin surface and inhibit the effect of the demineralized dentin matrix.

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Emdogain® (EMD) is made from enamel matrix proteins (EMPs) extracted from the permanent tooth germ of juvenile swine and propylene glycol alginate (PGA) as a matrix (1, 2). It contains mainly amelogenin; the remaining 10% of proteins include proline-rich nonamelogenins (3), tuftelin (4), tuft protein (5), serum protein (6, 7) and at least one salivary protein (7). EMPs are secreted from Hertwig's epithelial sheath during tooth development, and their function is to differentiate cells of the dental follicle into cementoblasts and to form acellular cementum (1, 8–10).

Experimentally, EMD stimulates cells of the periodontal ligament and increases alkaline phosphatase activity (11), cell proliferation (12–14), the secretion of transforming growth factor β1 (TGF-β1) and insulin-like growth factor 1 (11, 13) and calcification activity (14). Moreover, EMD also stimulates the proliferation of preosteoblastic 2T9 cells, inhibits the proliferation of osteoblast-like MG63 cells (15), increases alkaline phosphatase activity of MG63 cells (15), osteoblastic ST2 cells, osteoblastic KUSA/A1 cells (16) and by

mouse osteoblasts (17), increases the deposition of mineralized nodules by KUSA/A1 cells (16) and by mouse osteoblasts (17), and increases the production of osteocalcin and TGF-β1 by MG63 cells (15) and KUSA/A1 cells (16). Additionally, EMD induces the adhesion and the migration of fibroblasts, and accelerates their alkaline phosphatase activity and secretion of TGF-β1 (11) as well as their proliferation and matrix production (18). On the other hand, EMD inhibits epithelial cell division and differentiation (14, 19, 20), and elicits low immune responses by human peripheral lymphocytes, especially CD4 T-lymphocytes (21). *In vivo* studies showed that EMD accelerates the formation of acellular cementum (1, 22, 23) but inhibits the proliferation of epithelial cells (2). Recently, many studies reported that there was no difference when periodontal treatment was performed with or without EMD (24–27). Further, little is known about how EMD affects mesenchymal cells in extraosseous regions, such as muscle tissue.

Bone morphogenetic protein (BMP) was first reported by Urist in 1965 as a non-collagenous protein existing in demineralized bone and in the dentin matrix, which induces ectopic bone formation (28). Furthermore, the amino-acid sequence of BMP was characterized from bovine demineralized bone matrix in 1988 (29); BMP is 30–40% homologous to TGF-β in terms of amino-acid sequence and belongs to the TGF-β super family along with activin, inhibin and muellerian inhibitory factor. In addition, BMP is known to play important roles in cell proliferation, cell differentiation, regulation of apoptosis, early embryogenesis and angiogenesis *in vivo* and *in vitro* (28, 30–32).

Table 1. Experimental design. Each rat received three dentin tubes with Emdogain®, propylene glycol alginate or nothing, either in demineralized or in non-demineralized dentin tubes

		7day	14 day	21 day
Histological analysis	Demineralized	3 rats	3 rats	3 rats
	Non-demineralized	3 rats	3 rats	3 rats
Gene expression analysis	Demineralized	10 rats	10 rats	10 rats
	Non-demineralized	10 rats	10 rats	10 rats

The purpose of this study was to investigate whether EMD has the ability to induce osteogenesis when dentin matrix is implanted in muscle tissue with or without demineralization.

Material and methods

EMD and PGA were obtained from Seikagaku Corp., Tokyo, Japan. All experiments were performed in accordance with the Guidelines for Experimental Laboratory Animals in the animal facility of Tokyo Dental College.

Preparation of dentin tubes

Dentin tubes were prepared from rat incisors taken from maxilla and mandibles from 60 male Sprague Dawley rats, each weighing about 450 g, according to the method described by Inoue *et al.* in 1986 (31, 33). Briefly, the attached periodontal ligament and enamel matrix were removed from each tooth by mechanical means. Both ends of the incisors were then cut off using a band-saw and the dental pulps were removed. Each dentin tube was washed with a copious amount of water using a magnetic stirrer at 4°C for 12 h and was defatted with ethanol for 30 min and then with ether for 15 min. The dentin tubes were then divided into demineralized and non-demineralized groups. Dentin tubes of the demineralized group were demineralized with 0.6 N hydrochloric acid at room temperature for 3 h and BMP was expressed on the surface of each dentin tube, which was then washed with distilled water, followed by ethanol and ether for 10 min. The size of each dentin tube was approximately 8 mm in length.

Contents of dentin tubes

Two compositions were used in this study: (i) EMD, one syringe type and (ii) PGA as a solvent for EMD.

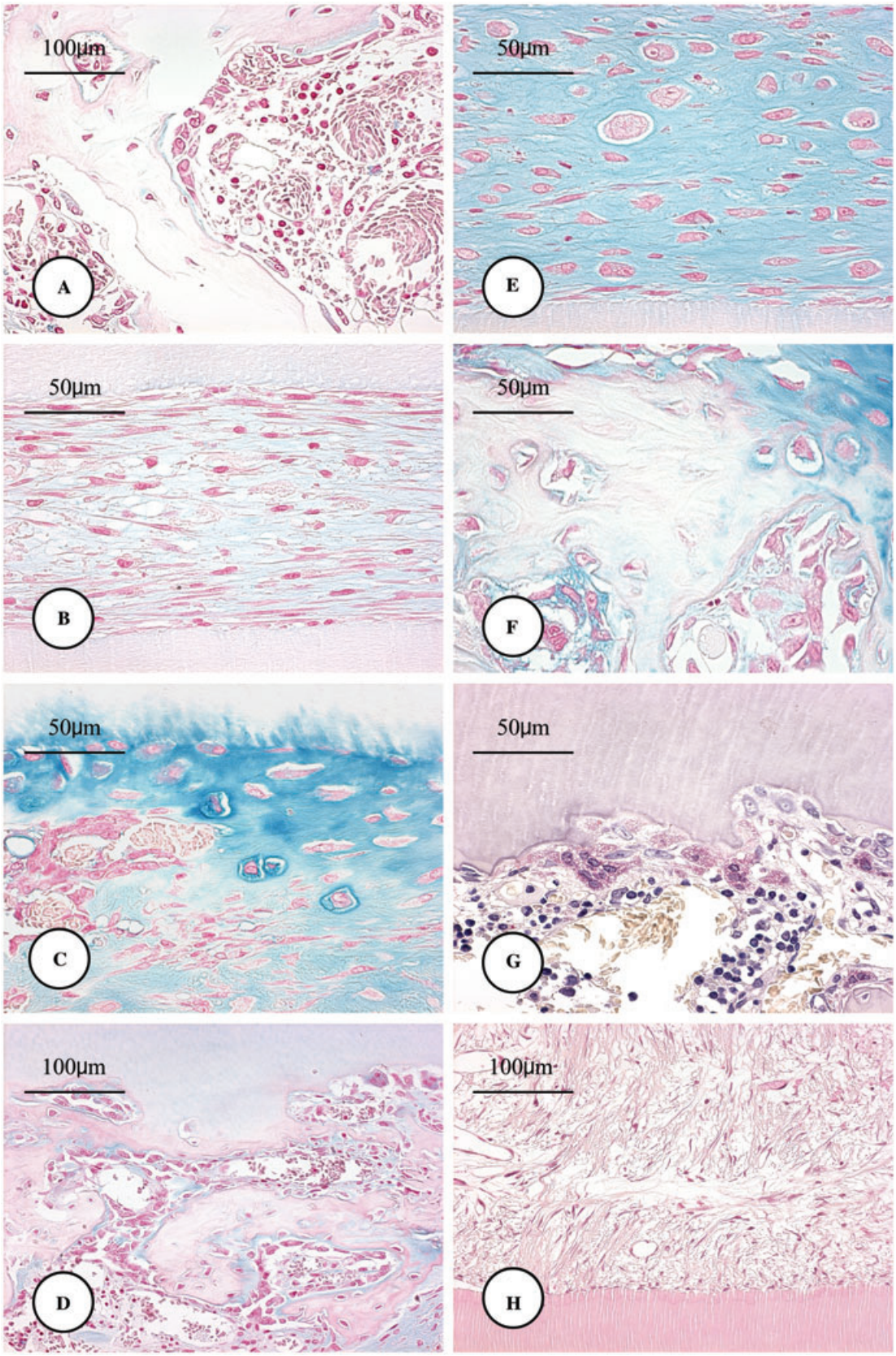
Preparation of grafts

The demineralized or non-demineralized dentin tubes were filled with approximately 10–15 μl of either EMD (EMPs + PGA) or PGA alone using a 23-gauge needle mounted on a syringe. Demineralized and non-demineralized dentin tubes without any contents were used as controls.

Transplantation

The dentin tubes of each group were transplanted into the rectus abdominal walls of 78 male Sprague Dawley rats, each weighing about 150 g (Table 1). Rats were anesthetized using 0.5 ml of 2.5% Ravonal® (Tanabe Seiyaku, Osaka, Japan). An incision approximately 2.0 cm in length was made in

Fig. 1 (overleaf). Histological images of transplanted dentin tubes. (a) At 21 days in the control group, the majority of cartilage had been replaced by trabecular bone tissue with bone marrow (Alcian-blue, demineralized group). (b) At 7 days in the Emdogain® (EMD) group, many fibroblast-like cells had invaded the dentin tubes (Alcian-blue, demineralized group). (c) At 14 days in the EMD group, cartilage was observed at the inner wall (Alcian-blue, demineralized group). (d) At 21 days in the EMD group, bone tissue with bone marrow was observed in the central area and osteoblasts and osteoclasts were observed on the margins of bone tissues and the dentin tube (Alcian-blue, demineralized group). (e) At 7 days in the propylene glycol alginate (PGA) group, chondromatrix with a few chondrocytes was observed in some areas (Alcian-blue, demineralized group). (f) At 14 days in the PGA group, most of the chondromatrix had already been replaced by bone tissue (Alcian-blue, demineralized group). (g) At 21 days in the PGA group, the inner walls of the dentin tubes were resorbed by tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts (TRAP, demineralized group). (h) At 21 days in the control group, cell-poor fibrous connective tissue was observed (hematoxylin–eosin, non-demineralized group).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the midline of the abdominal skin and three pockets were prepared on both sides of the rectus abdominal muscle using scissors. Three grafts were inserted into the pockets of the muscle using dental forceps, and the skin was sutured with silk thread.

Each rat received three dentin tubes with EMD, PGA or nothing, either in demineralized or in non-demineralized dentin tubes.

Histological observations

Three rats from each group were killed by an overdose of the anesthesia described above at each of 7, 14 and 21 days after the implantation. Muscle tissues with grafts were removed and fixed with 10% neutral buffered formalin solution. Non-demineralized dentin tubes were demineralized with 10% formic acid for 5 days. Specimens were dehydrated with a series of graded alcohols before being embedded in paraffin. Serial sections approximately 4–5 μm in thickness were cut parallel to the long axis of the dentin tube and were then stained with hematoxylin–eosin, alcian-blue or tartrate-resistant acid phosphatase (TRAP).

Morphometric analysis

Eight sections from each group were chosen from each of the time periods. The area of cartilage, chondromatrix and chondrocytes that was positive for alcian-blue and the area of bone that included osteoid and bone marrow tissues in each dentin tube were measured. The cartilage area and the bone area were divided by the total area of the inner aspect of the dentin tube and the cartilage ratio and the bone ratio were calculated. Analysis of variance and the multiple comparison Fisher's test were used to analyze the data.

Preparation of samples for quantitative reverse transcription–polymerase chain reaction using the LightCycler™

Ten rats from each group were killed by an overdose of the anesthesia solution described above at each of 7, 14 and 21 days after the surgical implantation (Table 1). Each dentin tube was homogenized using a Sonifier (Branson, Danbury, CT, USA) in trizol reagent (Invitrogen, Carlsbad, CA, USA) and total RNA was extracted. The chloroform suspension was centrifuged at 16,000 *g* for 20 min. Isopropyl alcohol was added to the supernatant, which was then frozen at −80°C overnight. Subsequently, each sample was centrifuged at 16,000 *g* for 20 min and the total RNA pellets were washed with 75% ethanol. Finally, the total RNA was dissolved in RNase-free water, and total RNA concentration was measured by absorbance using an UVmini-1240 (Shimadzu Corporation, Kyoto, Japan). Oligo dT primer (1 μl), dNTP (2 μl), RNase inhibitor (1 μl), reverse transcriptase (1 μl), 10 × buffer (2 μl) and $MgCl_2$ (4 μl) were added to total RNA (1 μg) and the volume was adjusted to 20 μl with RNase-free water. The mixture was used to synthesize cDNA by reverse transcription (42°C 15 min, 99°C 5 min) and stored at −80°C until analysis. Polymerase chain reaction (PCR) was then carried out using vascular endothelial growth factor (VEGF), osteopontin, osteocalcin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers. The primer sequences for VEGF from 5′ to 3′ were: forward, TACCAGCGCAGC TATTGCCGT; reverse, TTTGGTGA GGTTTGATCCGCATG. The primer sequences for osteopontin from 5′ to 3′ were: forward, CTCGGAGGAGAA GGCGCATTA; reverse, CCATCGT-CATCGTCGTCGTCA. The primer sequences for osteocalcin from 5′ to 3′ were: forward, GGTGCAAAGCC-CAGCGACTCT; reverse, GGAAGC-

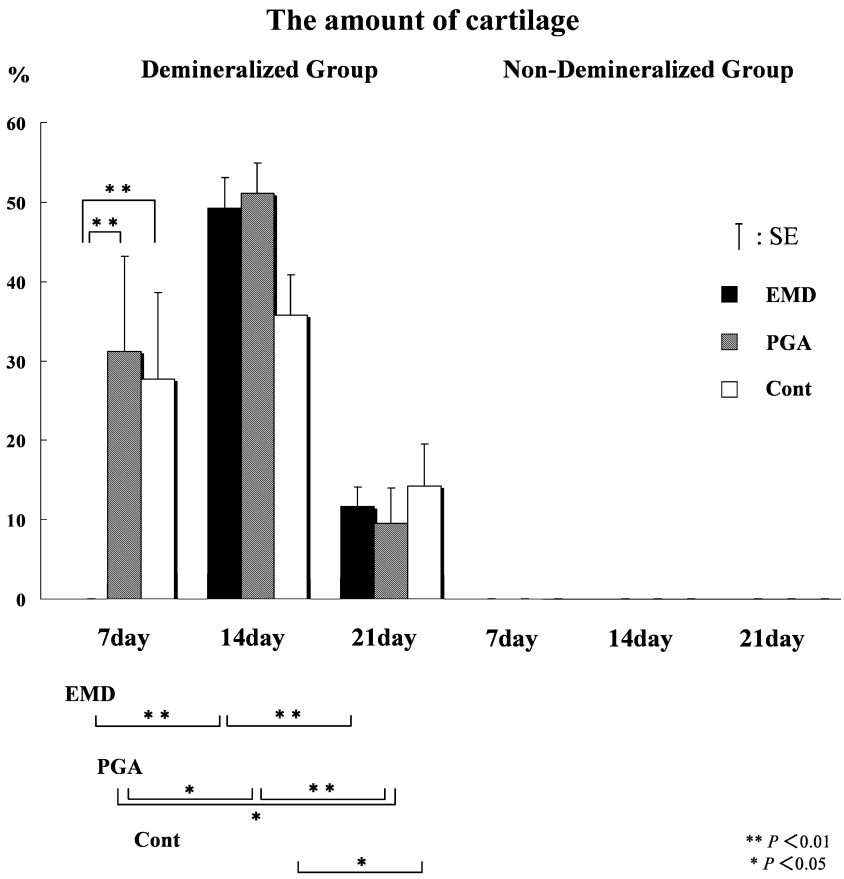

Fig. 2. Statistical analysis of the morphometric study. In the non-demineralized groups, cartilage and chondromatrix were not observed at any time. In the demineralized groups, the amount of cartilage in the Emdogain® group was delayed compared with the propylene glycol alginate and the control groups. EMD, Emdogain®; PGA, propylene glycol alginate; Cont, control.

CAATGTGGTCCGCTA. The primer sequences for GAPDH from 5′ to 3′ were: forward, TGAACGGGAAGCT CACTGG; reverse, TCCACCACCCT GTTGCTGTA. The amplification product sizes of VEGF, osteopontin, osteocalcin and GAPDH were 202, 207, 199 and 307, respectively. To measure mRNA levels, quantitative PCR assays were conducted with a LightCycler™ using the double-stranded DNA dye SYBR Green 1 (Roche Diagnostics GmbH, Manheim, Germany). Quantification was performed by comparing the levels obtained to a standardized sample. The VEGF, osteopontin, osteocalcin and GAPDH PCR conditions used in the LightCycler™ were 45 cycles (95°C 10 s, 54°C 5 s and 72°C 8 s), 45 cycles (95°C 10 s, 60°C 5 s and 72°C 12 s), 45 cycles (95°C 10 s, 60°C 5 s and 72°C 12 s) and 40 cycles (95°C 10 s, 60°C 10 s, 72°C 12 s), respectively. Melting curve analysis was also performed after the PCR amplification to confirm the absence of the primer dimer in the PCR products. The ratios of VEGF, osteopontin and osteocalcin mRNAs were adjusted by the value of GAPDH. The PCR products were separated on 2% agarose ethidium bromide gels. Analysis of variance and the multiple comparison Fisher's test were used to analyze the data.

Results

Microscopic observations

Demineralized group Control group: at 7 days after the transplantation, fibroblasts and capillaries had invaded the dentin tubes and chondrocytes with chondromatrix were observed. However, in the central part, the exudates were filled but cells were absent in the dentin tubes. At 14 days after the transplantation, capillaries had invaded the inner aspects of the dentin tubes and some of the calcified cartilage had been resorbed by TRAP-positive osteoclasts and was partially replaced by newly formed bone tissue. Resorption of the inner walls of the dentin tubes by TRAP-positive osteoclasts was also observed. At 21 days after the transplantation, the majority of cartilage had been replaced by trabecular bone tissue with bone marrow in the central part. The remodeling of both the newly formed bone tissue and the dentin tubes by TRAP-positive osteoclasts was frequently seen (Fig. 1a).

EMD group: at 7 days after the transplantation, many fibroblast-like cells had invaded the dentin tubes, however, neither cartilage nor bone tissues were observed (Fig. 1b). At 14 days after the transplantation, many capillaries were observed in the dentin tubes, cartilage was observed at the inner wall and newly formed bone tissues were observed attached to the dentin tubes (Fig. 1c). At 21 days after the transplantation, bone tissue with bone marrow was observed in the central area and large osteoblasts lined the margins of bone tissues. Resorption of bone tissue and dentin tubes by TRAP-positive osteoclasts was also observed (Fig. 1d).

PGA group: at 7 days after the transplantation, many fibroblasts had invaded the dentin tubes, and exudates were only observed in the central area. Some chondromatrix with a few chondrocytes was observed in some areas (Fig. 1e). At 14 days after the transplantation, most of the chondromatrix had already been replaced by bone tissue and many osteoblasts were observed in the margins of the bone tissue (Fig. 1f). At 21 days after the transplantation, bone tissue with bone marrow was observed. Remodeling of the bone tissue was evident and the inner walls of the dentin tubes were resorbed by TRAP-positive osteoclasts (Fig. 1g).

Non-demineralized group No induction of hard tissues was observed at any of the time periods in any of the groups.

Control group: at 7 days after the transplantation, fibroblasts had

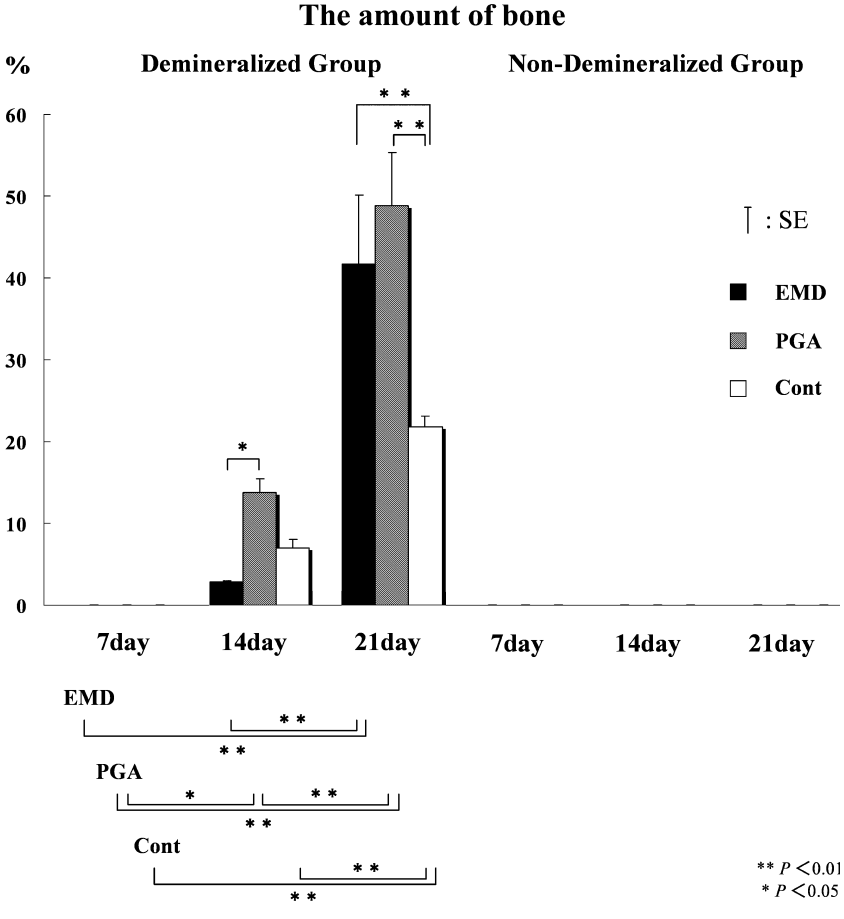

Fig. 3. Statistical analysis of the morphometric study. In the non-demineralized groups, bone and bone marrow were not observed at any time. In the demineralized groups, the amounts of bone and bone marrow were increased with time in all groups. EMD, Emdogain®; PGA, propylene glycol alginate; Cont, control.

invaded the dentin tubes and loose and edematous fibrous connective tissue with a number of lymphocytes and dilated capillaries were observed in the central area. At 14 days after the transplantation, the fibroblasts had invaded the central parts of the dentin tubes. At 21 days after the transplantation, cell-poor fibrous connective tissue was observed (Fig. 1h).

EMD group: At 7 days after the transplantation, cell-rich fibrous connective tissue was observed in the dentin tubes. At 14 days after the transplantation, the cell-rich fibrous connective tissue had become cell-poor fibrous connective tissue. At 21 days after the transplantation, the number of capillaries had decreased and the connective tissue had become hyaline degenerated.

PGA group: the features of this group were similar to those of the EMD group at each time period.

Morphometric analysis

Cartilage Demineralized group: at 7 days after the transplantation, there was no significant difference between the PGA and control groups, and at 14 days and 21 days after the transplantation, there were no significant differences among any of the groups (Fig. 2).

In the control group, there was significantly more cartilage at 14 days than at 21 days ($p < 0.05$) but there was no significant difference between 7 days and 14 days, and also there was no difference between 7 days and 21 days.

In the EMD group, the amount of cartilage was greater at 14 days compared with 21 days ($p < 0.01$).

In the PGA group, the amount of cartilage was greater at 14 days when compared with 7 days ($p < 0.05$) and 21 days ($p < 0.01$), and less cartilage was seen at 21 days than at 7 days ($p < 0.05$).

Bone tissue Demineralized group: at 14 days after the transplantation, there was significantly more bone tissue in the PGA group than in the EMD group ($p < 0.05$) but there was no significant difference between the control groups and EMD groups, and also there was no difference between the control groups and PGA groups at 7 days or at 21 days (Fig. 3).

At 21 days after the transplantation, the amount of bone tissue was the least in the control group ($p < 0.01$). However, there was no significant difference between the EMD group and the PGA group.

In the control group, the amount of bone tissue was the greatest at 21 days ($p < 0.01$) but there was no significant difference between 7 days and 14 days.

In the EMD group, the amount of bone tissue was the greatest at 21 days ($p < 0.01$) but there was no significant difference between 7 days and 14 days.

In the PGA group, the amount of bone tissue was the greatest at 21 days ($p < 0.01$) and there was significantly more bone tissue at 14 days than at 7 days.

mRNA expression

Vascular endothelial growth factor Control group: in the demineralized group, the expression of VEGF mRNA was the highest at 14 days ($p < 0.05$) but there was no significant difference between 7 and 21 days (Fig. 4).

EMD group: in the demineralized group, the expression of VEGF mRNA tended to increase with time ($p < 0.05$), but there was no significant difference among the various time periods.

PGA group: in the demineralized group, the expression of VEGF mRNA was the greatest at 14 days and was the least at 7 days ($p < 0.01$).

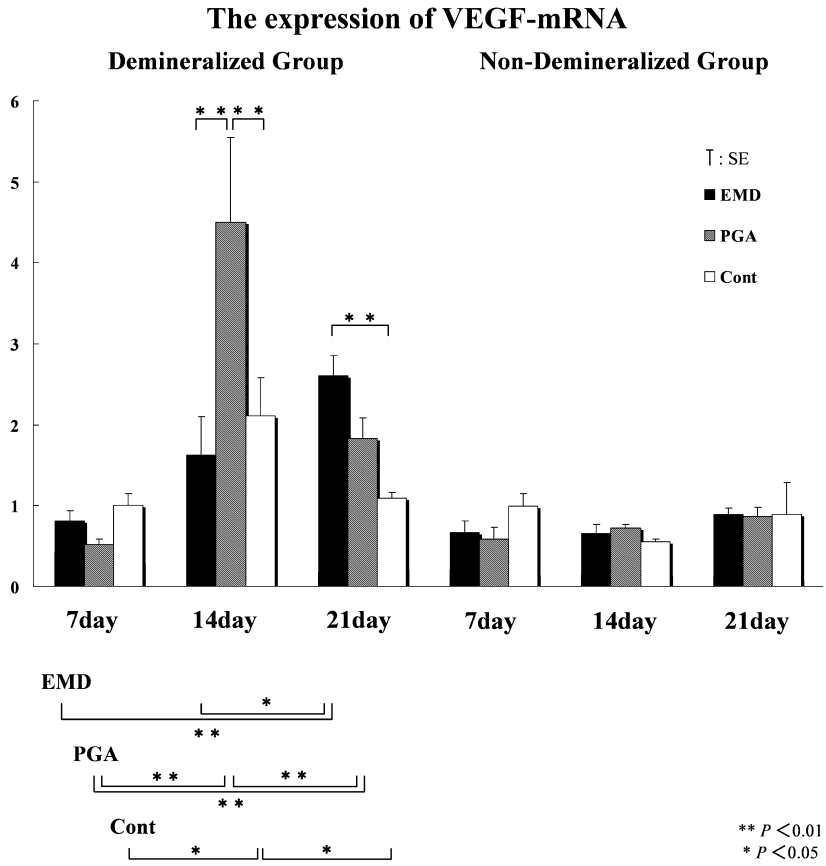

Fig. 4. The ratio of expression of vascular endothelial growth factor (VEGF) mRNA in demineralized and in non-demineralized dentin tubes. The expression of VEGF mRNA in the propylene glycol alginate group was the greatest at 14 days, and in the Emdogain® group at 21 days. In the non-demineralized groups, the expression of VEGF mRNA was not significantly different among any of the time periods in any of the groups. EMD, Emdogain®; PGA, propylene glycol alginate; Cont, control.

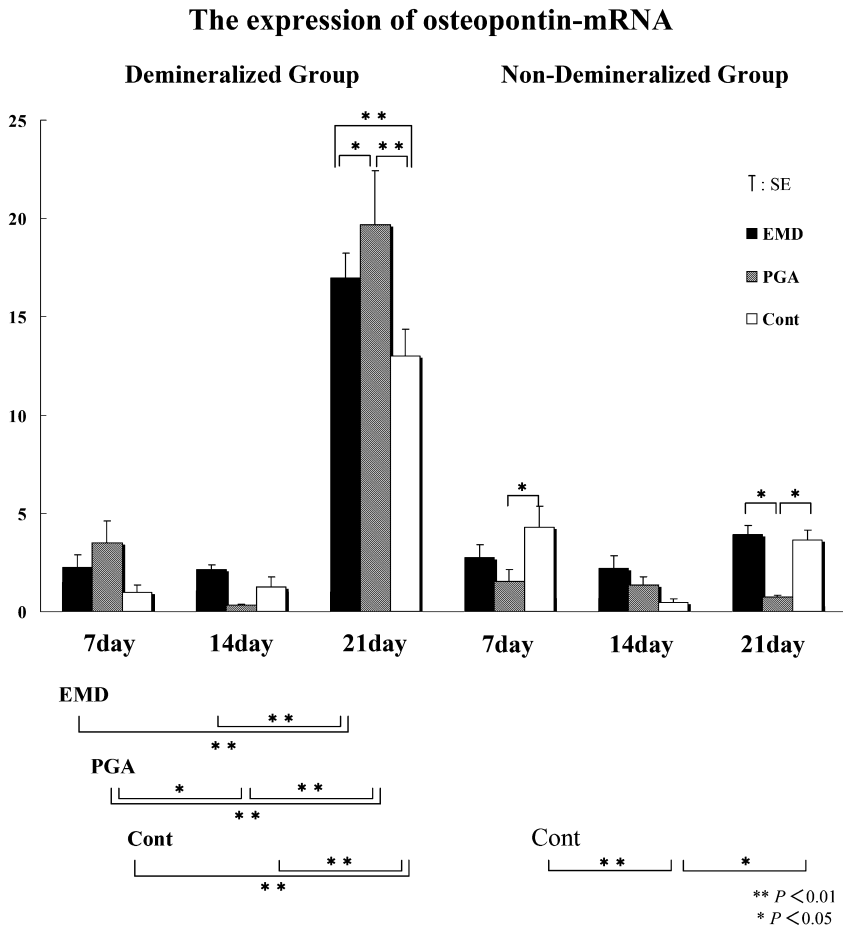

Fig. 5. The ratio of expression of osteopontin mRNA in demineralized and in non-demineralized dentin tubes. In the demineralized groups, the expression of osteopontin mRNA was the greatest at 21 days in all groups. EMD, Emdogain®; PGA, propylene glycol alginate; Cont, control.

In the non-demineralized group, the expression of VEGF mRNA was not significantly different among the various time periods.

Osteopontin Control group: in the demineralized group, the expression of osteopontin mRNA was the greatest at 21 days ($p < 0.01$), but there was no significant difference between 7 and 14 days. In the non-demineralized group, the expression of osteopontin mRNA was the least at 14 days ($p < 0.01$) but there was no significant difference between 7 and 21 days (Fig. 5).

EMD group: in the demineralized group, the expression of osteopontin mRNA was the greatest at 21 days ($p < 0.01$) but there was no significant difference between 7 and 14 days. In the non-demineralized group, there was no significant difference in osteopontin mRNA among the various time periods.

PGA group: in the demineralized group, the expression of osteopontin mRNA was the greatest at 21 days ($p < 0.01$). In the non-demineralized group, there was no significant difference among the various time periods.

Osteocalcin The expression of osteocalcin mRNA was not observed at any time period in the non-demineralized groups (Fig. 6).

In the demineralized groups, the expression of osteocalcin mRNA in each group was the greatest at 21 days ($p < 0.01$) but there was no significant difference between 7 and 14 days.

Discussion

Morphological observations

Recently, recombinant BMPs were detected and used in various experiments *in vitro*. However, the experimental system is not established to use recombinant *in vivo*. Demineralized dentin tube in which non-collagenous protein such as BMPs were contained were well established for induction studies. We decided that dentin tube must be a suitable material to observe the relationship between BMPs and EMD.

It is known that EMD influences the wound healing of periodontal tissue during periodontal regenerative therapy and the wound healing of pulp tissue during pulpectomy (1, 2, 22, 23, 34–36). The aim of those studies was to assess the influence of EMD on osteogenic tissues; however, only one study was found that used non-osteogenic tissue as a target. Boyan *et al.* reported that a porcine fetal enamel matrix derivative was not osteoinductive but was osteopromotive, due in part to its osteoconductive properties when implanted in mouse muscle (37). It is an open question whether EMD has a bone inductive capability or not.

It is also well known that ectopic endochondral ossification in various tissues is induced by demineralized dentin containing BMP (28, 31, 33). In this study, endochondral ossification occurred in all of the demineralized groups, but not in any of the non-demineralized groups. These results show that neither EMD nor PGA has an osteoinductive capability. Endochondral ossification in the EMD groups was delayed compared to both the PGA and the control groups in the demineralized group. Gestrelius *et al.* reported that the viscosity of PGA changed depending on the pH and temperature and decreased under physiological conditions (38). After the application of EMD to the surface of replanted teeth, EMPs in EMD were released from the PGA and aggregated on the root surface (38). The aggregated EMPs gradually disappeared from the root surface within 7 days after the application of EMD on the surface of

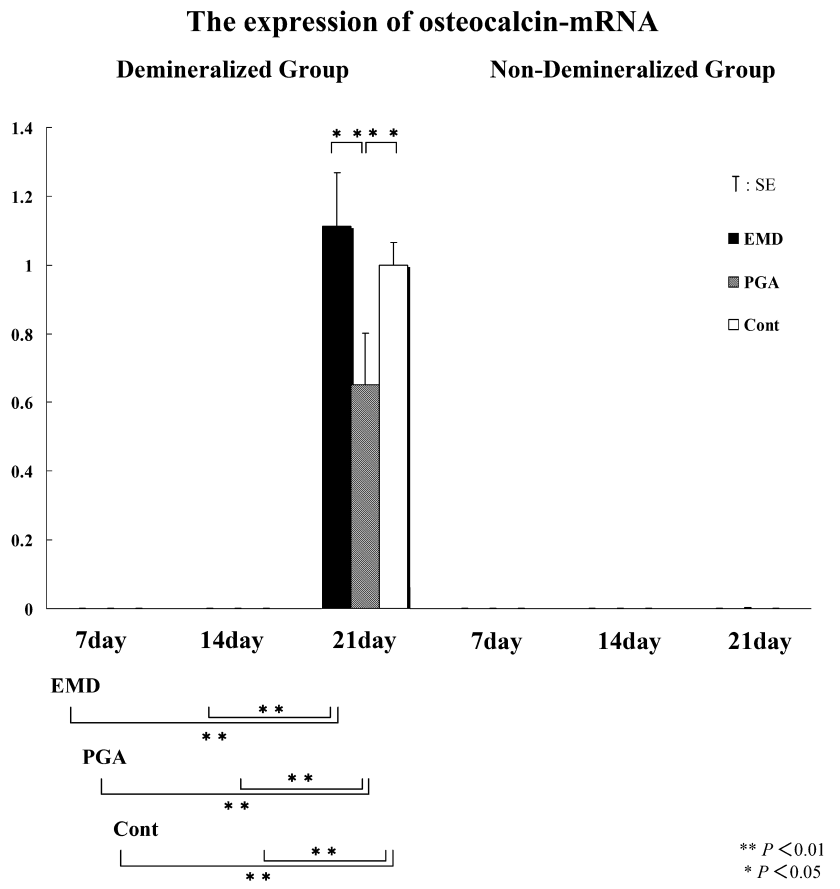

Fig. 6. The ratio of expression of osteocalcin mRNA in demineralized and in non-demineralized dentin tubes. In the demineralized groups, expression of osteocalcin mRNA was the greatest at 21 days. However, in the non-demineralized group, the expression of osteocalcin mRNA was not observed at any time period. EMD, Emdogain®; PGA, propylene glycol alginate; Cont, control.

the replanted teeth (38). This fact suggests that EMPs in the demineralized dentin tube are released from the PGA and aggregate on the surface of the inner aspect of the demineralized dentin matrix, that EMPs inhibit the effect of demineralized dentin matrix until 7 days after the implantation, and that chondrogenesis in the EMD group in the demineralized group did not occur. Furthermore, EMPs were usually applied at 150–200 μg/ml as a maximum concentration; however, ready-made EMD at a concentration of 30 mg/ml as used in these experiments is too high a concentration for the cells to undergo osteoinduction.

Hakki *et al.* reported that EMD could accelerate the proliferation and regulate the activity of dental follicle cells in murine follicles (39). Moreover, Schwartz *et al.* reported that EMD stimulates the proliferation but not the differentiation of immature osteoblasts, and inhibits the proliferation but stimulates the differentiation of mature osteoblasts *in vitro* (15). This suggests that EMD in the demineralized dentin tube promotes the proliferation of mesenchymal cells until 7 days after the implantation, and that these proliferating cells differentiate into chondrocytes due to the exposed demineralized dentin matrix surface, as EMPs might have disappeared from the surface of the demineralized dentin at 7 days after the implantation.

mRNA expression

Vascular endothelial growth factor (VEGF) is a secretary glycoprotein, which induces angiogenesis and which is produced by perivascular smooth muscle cells and pericytes (40, 41). Recently, Gerber *et al.* reported that VEGF is produced by hypertrophic chondrocytes (42). During events of endochondral ossification, chondrocytes degenerate and become hypertrophic chondrocytes, after which capillaries invade them and ossification occurs. In those experiments, hypertrophic chondrocytes were first observed at 14 days in the PGA groups of the demineralized group and expression of VEGF mRNA was detected at 14 days and was the greatest compared to the other groups. On the other hand, hypertrophic chondrocytes first appeared at 21 days in the EMD groups of the demineralized group and the expression of VEGF mRNA was higher than in the PGA groups at 21 days. These results suggest that the endochondral ossification and the expression of VEGF mRNA were delayed in the EMD group, as demineralized dentin matrix is masked by EMPs at 7 days.

Osteopontin is a protein that has a molecular structure made from approximately three hundred amino acids (43) and which is involved with cell adhesion (44). Osteopontin is produced by hypertrophic chondrocytes (45) preosteoblasts, osteocytes, osteoclasts (46–48), osteoblasts, chondrocytes, fibroblasts (49) and macrophages (50). However, osteopontin is the most abundant protein produced by cells related to osteogenesis, such as osteoblasts or osteocytes, and osteopontin expression usually appears before osteocalcin expression during osteogenesis (51, 52). The expression of osteopontin mRNA at 7 days and at 14 days was less than that produced at 21 days in all of the demineralized groups, because the cells invading from the muscle into the dentin tubes were not sufficiently mature as osteoblasts at 7 and 14 days. The highest expression of osteopontin mRNA was observed at 21 days in all demineralized groups, and these results suggest that the majority of cells in the dentin tubes had differentiated into osteoblasts at 21 days in the demineralized group, particularly in the PGA group. The calcified matrix was seen after 14 days and started to be resorbed by osteo-

clasts in the process of enchondral ossification. Furthermore, resorption of the peripheries of the dentin tubes also started at this stage. Osteoclasts that had appeared at this time probably influenced the highest expression of osteopontin mRNA at 21 days.

Osteocalcin is a bone-specific protein produced by mature osteoblasts and is used as a marker of calcification in osteogenesis (53). Osteocalcin is required for osteogenesis and also has an inhibitory function against excess calcification (54). The expression of osteocalcin mRNA increased with time in all demineralized groups. The expression of osteocalcin mRNA in the PGA group at 21 days was less than in the EMD or control groups. This suggests that the peak of expression of osteocalcin mRNA in the PGA group had already passed the stage of osteogenesis and the osteogenesis had shifted to the resorption phase at 21 days.

In conclusion, the results of this study suggest that neither EMD nor PGA have the ability to induce hard tissue and that EMPs delay the ability of demineralized dentin matrix, which exists on the surface of the dentin tubes. Thus, PGA is a proper material for cells to migrate into dentin tubes.

Acknowledgements

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