J Periodont Res 2004; 39; 249–256
Printed in the UK. All rights reserved

Copyright © Blackwell Munksgaard Ltd

JOURNAL OF PERIODONTAL RESEARCH
doi: 10.1111/j.1600-0765.2004.00733.x

Effect of heat treatment on bioactivities of enamel matrix derivatives in human periodontal ligament (HPDL) cells

Takatoshi Nagano[1], Takanori Iwata[3], Yorimasa Ogata[4], Takako Tanabe[2], Kazuhiro Gomi[1], Makoto Fukae[2], Takashi Arai[1], Shinichiro Oida[2]

Department of [1]Periodontics and Endodontics, and [2]Biochemistry, School of Dental Medicine, Tsurumi University, Tsurumi-ku, Yokohama, Japan, [3]Section of Periodontology, Department of Hard Tissue Engineering, Graduate School, Tokyo Medical & Dental University, Bunkyo-ku, Tokyo, Japan and [4]Department of Periodontology, Nihon University School of Dentistry at Matsudo, Chiba, Japan

Nagano T, Iwata T, Ogata Y, Tanabe T, Gomi K, Fukae M, Arai T, Oida S. Effect of heat treatment on bioactivities of enamel matrix derivatives in human periodontal ligament (HPDL) cells. J Periodont Res 2004; 39; 249–256.
© Blackwell Munksgaard, 2004

Objectives: It has been shown that Emdogain® (EMD) containing enamel matrix derivative has cementum- and osteo-promotive activities *in vivo* and *in vitro*. Nevertheless, the commercial sale of EMD was halted because it has some possible risk for infectiosity. At present, Emdogain® Gel (Emd-Gel) containing the EMD heated to avoid the infectiosity is commercially available. The purpose of this study was to compare the *in vitro* bioactivities of Emd-Gel and EMD.

Material and methods: Healthy human periodontal ligament (HPDL) cells were used to study the effect of Emd-Gel and EMD on cell differentiation. The HPDL cells exposed to Emd-Gel and EMD were evaluated for the following effects: (i) alkaline phosphatase (ALP) activity; (ii) mitogenic (MTT) assay; (iii) biomineralization activity; (iv) gene expressions using reverse transcription-polymerase chain reaction (RT-PCR). The effect of EMD with or without heat treatment was examined for ALP activity on the cell differentiation.

Results: The effect of Emd-Gel on ALP activity was greater than that of EMD. It was confirmed from the effect of EMD with heat treatment at 60°C, 80°C and 100°C on the ALP activity. The effect of Emd-Gel on the biomineralization activity was also greater than that of the EMD. The Emd-Gel has a stronger effect for the expression of osteoblast-like phenotype than the EMD.

Conclusion: The results indicate that the Emd-Gel has greater bioactivities than the EMD *in vitro*.

Takatoshi Nagano, Department of Periodontics and Endodontics, School of Dental Medicine, Tsurumi University, 2-1-3 Tsurumi, Tsurumi-ku, Yokohama 230–8501, Japan
Fax: +81 45 573 9599
e-mail: nagano-takatoshi@tsurumi-u.ac.jp

Key words: differentiation; Emdogain Gel; enamel matrix derivative; human periodontal ligament cells

Accepted for publication February 2, 2004

It is known that enamel extracts have bioactivities such as the induction of osteogenesis (1, 2) and cementogenesis (3–5). For example, it is shown in *in vivo* and *in vitro* systems that the Emdogain® (EMD) have cementum- and osteo-promotive activities (6) and stimulate the proliferation and differentiation of osteoblastic cell (7–11).

In developing dental enamel, there are five protein species, which contain three matrix proteins and two proteinases. These are already cDNA cloned from the developing tooth germs of bovine, human, pig, rat, mouse, etc. The three matrix proteins are amelogenin (12), enamelin (13), and sheathlin (14, 15), also known as ameloblastin (16) or amelin (17). The two proteinases are enamelysin (18) and KLK4 which is also known as EMSP-1 (19). Ameloblastin and amelin, first cloned from rat-tooth specific cDNA libraries, are homologues of porcine sheathlin.

It has been reported recently that porcine enamel extracts contain bone morphogenetic proteins (BMPs) (2),

and the EMD has transforming growth factor-beta (TGF-β)-like activity in both oral epithelial and fibroblastic cells (20). Some members of the BMP family, which belonged to the TGF-β super family (21), can induce osteogenesis *in vivo* (22) and osteogenic differentiation *in vitro* (23).

Although there is increased evidence on EMD related to bone formation (1, 2) or periodontal regeneration (3–5), the active material in enamel extracts and the mechanisms involved in these processes are not well understood.

Currently the commercial sale of EMD has been stopped in Japan, because it has some possible risk for infectiosity. The Emdogain® Gel (Emd-Gel) is supplied with a premixed type commercially available now. According to the manufacturer's manual, the difference between Emd-Gel and EMD is the heat treatment of proteins to avoid the infectiosity.

Clinical research has demonstrated recently that the Emd-Gel gives equal clinical outcomes compared with EMD in the treatment of intrabony periodontal defects (24). The aim of this study was to compare the bioactivities of Emd-Gel and EMD *in vitro*.

Material and methods

Material

Emd-Gel (lot number: ETP082), EMD (lot number: 961971), and propylene glycol alginate (PGA) (control) (Biora, Malmo, Sweden) were generously supplied by Seikagaku-kougyo Corporation, Tokyo, Japan.

Preparation of EMD and Emd-Gel

Since the EMD was supplied with a set of lyophilized EMD (30 mg) and PGA solution (1 ml), these were mixed according to the manual prior to use (25). The Emd-Gel was supplied with a premixed type in which heat-treated EMD is already dissolved in PGA solution. According to the manual, the Emd-Gel contained 30 mg of enamel proteins per 1 ml of PGA solution. Samples were prepared by adding 29 ml of ultrapure water to the Emd-Gel or the mixed EMD to gain 1 mg/ml of solution. The PGA was diluted to obtain the same concentration in the sample as a control.

To evaluate the effect of the heat treatment, the mixed EMD samples (1 mg/ml) were separately heat treated at 60°C, 80°C and 100°C for 2 h. Each heat-treated EMD was measured for alkaline phosphatase (ALP) activity according to the method described below.

Cell culture

Three periodontally healthy premolars were collected from three patients who had undergone extraction for orthodontic reasons. Informed consent was obtained from all patients under a protocol approved by the Ethics Committee of Tsurumi University. Human periodontal ligament (HPDL) cells were obtained as previously described by Somerman *et al.* (26, 27). The cells were maintained in the alpha modification of Eagle's medium (αMEM; Life Technologies, Grand Island, NY, USA) containing 10% fetal bovine serum (Asahi Technoglass, Chiba, Japan) and 1% antibiotics [100 U/ml of Penicillin-G and 100 μg/ml of Streptomycin sulfate (Gibco BRL, Grand Island, NY, USA)] at 37°C in a humidified 5% CO_2 atmosphere.

Cell proliferation (mitogenic assay)

The mitogenic activity of the samples was assayed using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) (Sigma, St.Louis, MO, USA) according to the manufacturer's instructions. The HPDL cells were incubated in 100 μl of growth medium at an initial density of 3×10^3 cells/well. After 24 h of incubation, the αMEM containing 1.0% fetal bovine serum and 50 μg/ml of samples was then changed. After 96 h, 10 μl of MTT (5 mg/ml) was added to each well, and the cells were incubated for 4 h. The medium was then discarded, and 100 μl of dimethylsulfoxide was added to each well. The absorbance of each well was measured at 570 nm with background subtraction at 655 nm using a microplate reader (Bio-Rad Model 450, Hercules, CA, USA).

Alkline phosphatase activity (enzyme assay)

The HPDL cells were distributed in 96-well plates at a density of approximately 5×10^5 cells/well and incubated for 24 h. The growth medium was then changed to contain 10 nM of 1α,25-dihydroxyvitamin D_3 (Calbiochem, La Jolla, CA, USA) and 50 μg/ml of samples dissolved in ultrapure water. After 96 additional hours of incubation, the cells were washed once with phosphate-buffered saline, and ALP activity was determined using 10 mM *p*-nitrophenylphosphate as the substrate in 100 mM 2-amino-2-methyl-1,3-propanediol-HCl buffer (pH 10.0) containing 5 mM $MgCl_2$ and incubated for 10 min at 37°C. Adding NaOH quenched the reaction, and the absorbance at 405 nm was read on a plate reader.

Mineralization activity (differentiation assay)

The HPDL cells were plated in 24-well plates at an initial density of 3×10^4 cells/well. After 24 h of incubation, the medium was replaced with growth medium containing 50 μM ascorbic acid, 10 mM β-glycerophosphate, and 10 nM 1α,25-dihydroxyvitamin D_3 (differentiation medium) and 25 μg/ml of samples. The medium was changed every 72 h. The cells were maintained for 15 days and then the medium was discarded.

The compartments of cells were fixed in 100% methanol, stained with alizarin red S for 10 min, then washed with ultrapure water and photographed to examine the biomineralization activity. The staining solution was 1% alizarin red S (sodium alizarin sulfonate) (Sigma) dissolved in ultrapure water and adjusted to pH 6.4 with 0.1 N ammonium hydroxide.

For measuring the calcium content, the compartments of cells were dissolved by 0.5 N hydrochloric acid. The resulting solution was measured by a Calcium C-test kit and protocol (Wako Pure Chemical Industries Ltd, Osaka, Japan). The absorbance at 570 nm was read on a plate reader.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using RNAzol™ B (Tel-Test Inc., Friendswood, TX, USA) from the HPDL cells that were cultured in differentiation medium by adding 50 μg/ml of samples for 1, 4, 7, and 10 days. In some cases, the cells were cultured in the differentiation medium without ascorbic acid or β-glycerophosphate. The cDNA was synthesized from 3 μg of the total RNA using an oligo-dT primer and the You-primed First-Strand Beads kit (Amersham-Pharmacia Biotech, Piscataway, NJ, USA) according to the manufacturer's protocol. PCR primers were designed, based upon homosapiens mRNA sequences. The primer pairs were as follows: alkaline phosphatase (ALP), 5′-AGAGGGCCACGAAGG GGAACT-3′ and 5′-GGACGGACCC TCGCCAGTGCT-3′ (425 bp); osteo-

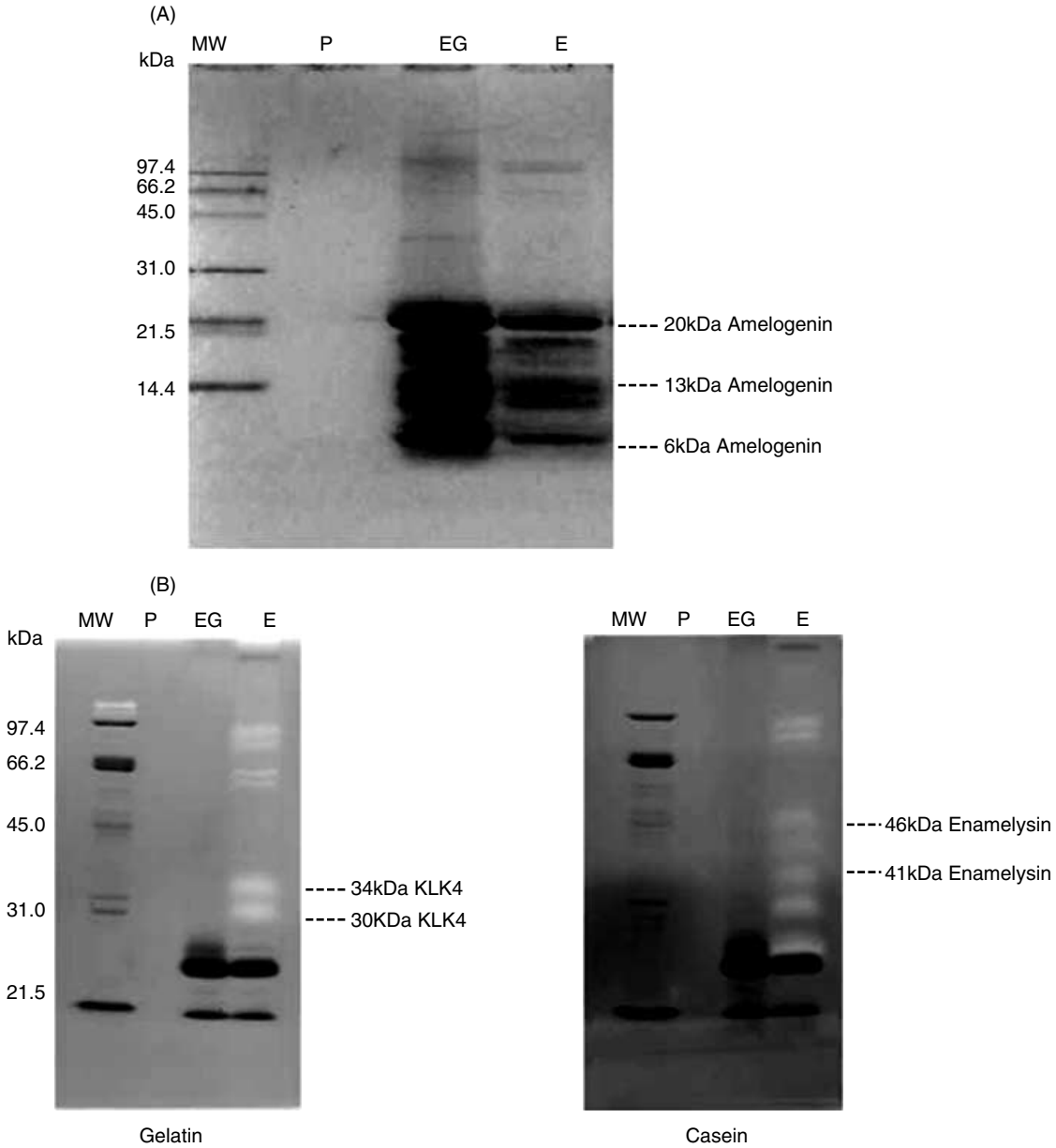

Fig. 1. (A) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis profiles of Emdogain® Gel (Emd-Gel), Emdogain® (EMD) and propylene glycol alginate (PGA) stained with Coomassie Brilliant Blue. Molecular weights (Bio-Rad Low Range Standards, Hercules, CA, USA) are shown in the left margin. The Emd-gel and EMD exhibited the same protein profile. However, Emd-gel has more protein contents than EMD. (B) Gelatin and casein zymograms showing the relative proteolytic activities in equal volumes of samples. The gel of casein zymogram was incubated in 2 mM calcium. In the EMD, proteinase activities such as KLK4 and enamelysin were detected, whereas no proteinase activity was found in the Emd-Gel and PGA. P, PGA; EG, Emd-Gel; E, EMD; MW, molecular weight markers.

pontin (OPN), 5′-TGACCTCTGTG AAAACAGCGT-3′ and 5′-TGTACA TTGTGAAGCTGTGAA-3′ (301 bp); osteocalcin (OC), 5′-TTGTGTCCA AGCAGGAGGGCA-3′ and 5′-ACA TCCATAGGGCTGGGAGGT-3′ (304 bp); bone sialoprotein (BSP), 5′-GCAGAAGTGGATGAAAACGA-3′ and 5′-TGGTGGTAGTATTCTGAC-CA-3′ (448 bp). A primer set amplifying glyceraldehyde-3-phosphate dehydrogenase (Clontech, Palo Alto, CA, USA) was used as a control. PCR conditions (Perkin-Elmer/GeneAmp PCR system 9600) were as follows: PCR started with a 10-min denaturation at 94°C, followed by 25 cycles with denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s, and product extension at 72°C for 30 s; in the final cycle, the 72°C extension lasted 7 min. PCR products were analyzed by 4.5% polyacrylamide gel electrophoresis in Tris-borate-EDTA (TBE) buffer (pH 8.0). Gels were stained with ethidium bromide, and the bands were visualized under UV light. Additionally, the PCR products were cloned into pBluescriptIISK(+) (Stratagene, La Jolla, CA, USA), and their nucleotide sequences were determined by cycle sequencing.

Analytical methods

Sodium dodecyl sulfate–polyacrylamide gel electrophoreses (SDS-PAGE) were carried out using 15% polyacrylamide slab gels containing 1% sodium dodecyl sulfate (SDS) according to the method of Laemmli, and the gels were stained with Coomassie Brilliant Blue R-250 (28).

Zymographies were performed using acrylamide gels containing 1% SDS and 0.033% gelatin or α-casein (Sigma) as a substrate according to the method of Heussen and Dowdle (29). Gelatin and α-casein zymograms showed the detection of KLK4 (19) and enamelysin (18), respectively.

Statistical analysis

All values are represented as means ± standard error (SE). Statistical significance was determined using an unpaired Student's *t*-test, and $p < 0.05$ was considered statistically significant.

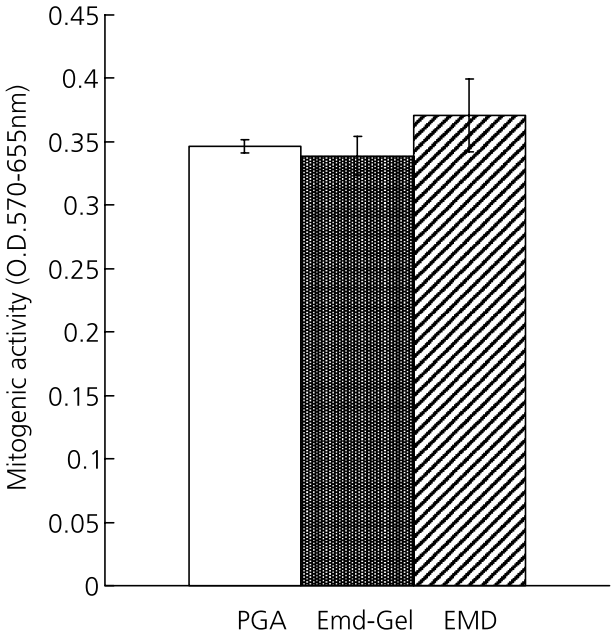

Fig. 2. The mitogenic activity was evaluated by MTT assay after 96 h of incubation. Data are presented as the mean ± SE ($n = 3$). There were no significant differences between the samples. EMD, Emdogain®; Emd-Gel, Emdogain® Gel; PGA, propylene glycol alginate.

Results

Differences of protein profiles

The SDS-PAGE profiles of the Emd-Gel, EMD and PGA are shown in Fig. 1(A). From the SDS-PAGE profiles, 20-, 13-, and 6 kDa amelogenins were contained mainly in the Emd-Gel and EMD (30), whereas no protein band was detected in the PGA. The Emd-gel and EMD exhibited the same protein profile. However, Emd-gel has more protein contents than EMD. No difference in the protein profiles of the EMD with or without heat treatment was detected on the SDS-PAGE (data not shown).

We compared proteolytic activities at Emd-Gel, EMD and PGA by zymography (Fig. 1B). KLK4 activity, which corresponds to 30- and 34 kDa bands, was detected in the EMD sample, and was not detected in the Emd-gel and PGA samples. Enamelysin activity, which corresponds to 41- and 46 kDa bands, was detected in the EMD, and was not detected in the Emd-gel and PGA samples.

Effect of proliferation

The growth-stimulative effect of Emd-Gel and EMD on the HPDL cells was examined by MTT assay. Both samples at concentrations of 50 μg/ml did not show any growth-stimulative effect (Fig. 2).

Comparison of alkaline phosphatase activity

The ALP activity of the HPDL cells was stimulated by Emd-Gel and EMD (Fig. 3). Their ALP activities increased dependent on the doses of both Emd-Gel and EMD. The changes occurring in the ALP activity by adding the Emd-Gel were always 1.7-fold greater than those occurring by adding the EMD.

Mineralization activity

When the compartment of HPDL cells were stained with alizarin red S at the end of the experimental period (day 15), many mineralized nodules were distinctly stained in the Emd-Gel compared with EMD and PGA. The calcium contents were also increased by Emd-Gel and EMD. The result

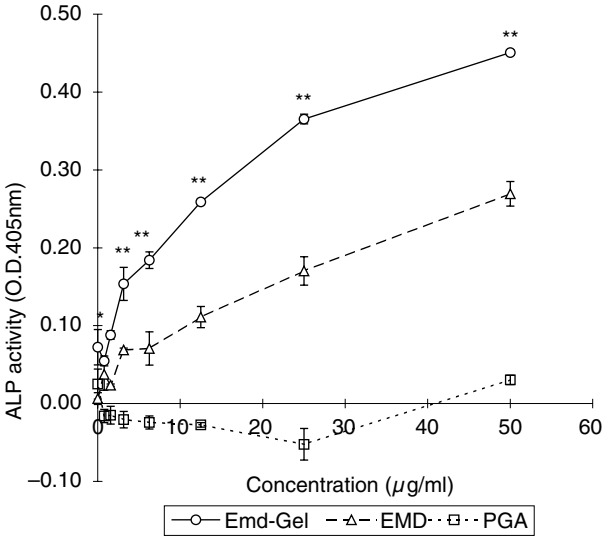

Fig. 3. The alkaline phosphatase (ALP) activity of human periodontal ligament (HPDL) cells induced by Emdogain® Gel (Emd-Gel) and Emdogain® (EMD). Emd-Gel induced 1.7-fold higher ALP activity than EMD. Data are means ± SE of three culture wells. Significantly different from EMD at $*p < 0.05$; $**p < 0.01$.

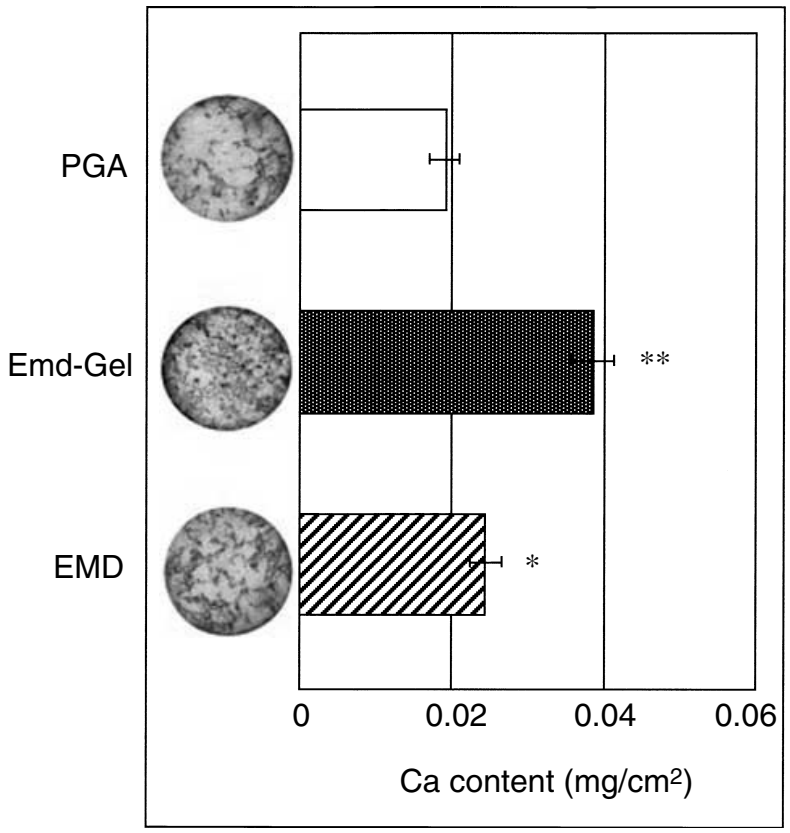

Fig. 4. The activity of mineralization induced by Emdogain® Gel (Emd-Gel) and Emdogain® (EMD). The mineralized nodules were stained with alizarin red S and are shown in the left circles, and calcium contents are shown by the right bars. Data are means ± SE of three culture wells. There were significant differences between propylene glycol alginate (PGA), Emd-Gel ($**p < 0.01$), and EMD ($*p < 0.05$) on calcium content.

shows that the calcium content stimulated by the Emd-Gel was almost 1.7-fold higher than that resulting from the addition of EMD (Fig. 4).

Gene expressions of tissue-specific differentiation markers for osteoblasts

RT-PCR was carried out to determine the gene expression of tissue-specific differentiation markers for osteoblasts, such as ALP, OPN, OC, and BSP. The RT-PCR results showed that ALP expression was increased at day 1 in the presence of Emd-Gel, and OPN expression was increased at day 4. The OC expression was increased at day 10 in the presence of Emd-Gel, and that of BSP was increased in the presence of Emd-Gel at day 10 (Fig. 5). The up-regulations were also confirmed in the gene expression of ALP, OPN, OC, and BSP within 24 h when the cells were cultured in the medium without ascorbic acid or β-glycerophosphate.

Effect of the heat-treated EMD on the alkaline phosphatase activity

We examined the ALP activity to evaluate the effect of heat-treated EMD. The result shows that the EMD with heat treatment at 60°C, 80°C and 100°C stimulated the ALP activities by almost two-fold greater than the EMD without heat treatment (Fig. 6).

Discussion

The enamel matrix derivative is clinically used as the EMD expecting the induction of cementogenesis along the root surface of the tooth (31, 32). However, the commercial sale of EMD has been stopped for possible risk of infectiosity. At present the Emd-Gel, which contains the EMD heat treated for avoiding the infectiosity, is commercially available. Therefore, a comparison of the *in vitro* bioactivities of Emd-Gel with EMD was carried out on the HPDL cells culture system.

In this study, it was demonstrated that Emd-Gel more enhanced the ALP activity of HPDL cells than the EMD. It was also shown that Emd-Gel more induced the mineralization activity

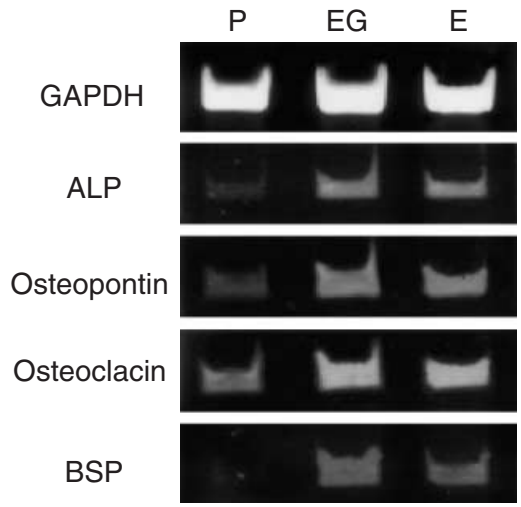

Fig. 5. The mRNA expression of alkaline phosphatase (ALP), osteopontin, osteocalcin, and bone sialoprotein (BSP) on HPDL cells cultured with 50 μg/ml of samples. Total RNA of HPDL cells incubated with Emdogain® Gel (Emd-Gel), Emdogain® (EMD) and propylene glycol alginate (PGA) was collected on days 1, 4, 7, and 10. Each culture was incubated with 50 μM ascorbic acid, 10 mM β-glycerophosphate, and 10 nM 1α,25-dihydroxyvitamin D_3. Each PCR product was based on an equal amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and separated by electrophoresis on a 4.5% polyacrylamide gel. Emd-Gel increased the expression of ALP at day 1, OPN at day 4, OC at day 10, and BSP at day 10. P, PGA; EG, Emd-Gel; E, EMD.

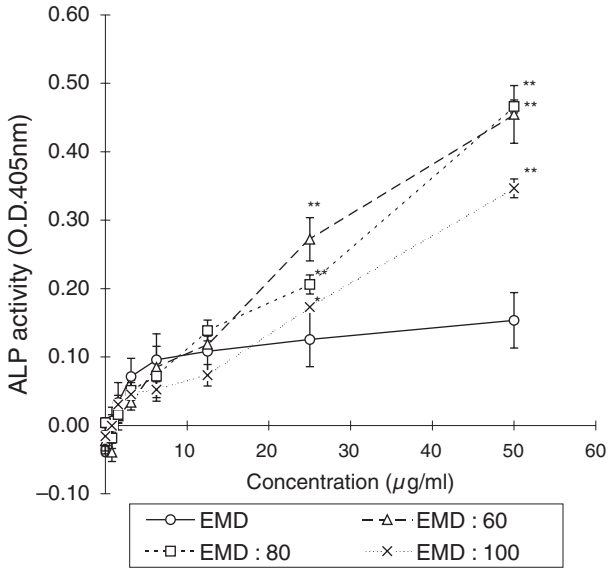

Fig. 6. The alkaline phosphatase (ALP) activity of Emdogain® (EMD) with heat treatment. EMD was separately heat-treated at 60°C, 80°C, and 100°C, for 2 h. The ALP activity of EMD with heat treatment at 60°C, 80°C and 100°C was two-fold higher than EMD without heat treatment. Data are means ± SE of three culture wells. Significantly different from without heat treatment EMD at $*p < 0.05$; $**p < 0.01$.

when cultured in the differentiation medium and more up-regulated gene expressions of tissue-specific differentiation markers for osteoblasts. We also confirmed the up-regulations in the expression of these markers' mRNAs when the cells were cultured in the medium without ascorbic acid or β-glycerophosphate. On the other hand, there were no significant differences of cell proliferation between Emd-Gel and EMD. This result indicate that the growth factor of induced proliferation response is not inactivated after heat treatment.

In this study, it was very important to measure the Emd-gel and EMD protein contents. We confirmed the protein contents by using automatic amino acid analyzer (JEOL JLC-500). The protein contents of Emd-gel were 1.5 times as much as that of EMD from the amino acid analysis. These results indicate that the Emd-Gel has a stronger effect for the expression of osteoblast-like phenotype and biomineralization activity than EMD on the HPDL cells cultured system, even if the protein content of Emd-Gel, which is 1.5 times as much as that of EMD, is taken into consideration from amino acid analysis.

The HPDL cells cultured from explants consist of heterogeneous populations, which included the periodontal mesenchymal cells (33, 34). Little information has been reported with regard to the particular effect of EMD on the cell differentiation of pluripotential mesenchymal cells, whereas the effects of osteoblastic differentiation at various maturation stages has been studied using several osteoblast-like cells (8–11).

Concerning the osteoinductive ability, BMPs are members of the TGF-β super family (21), which have been discovered recently in the EMD (2). These active proteins, which have been purified from bovine bone and cloned as BMPs (35, 36), are known sources of osteoinduction *in vivo* (22) and *in vitro* (23). Recently, it has also been reported that the EMD has TGF-β-like activity on both oral epithelial and fibroblastic cell cultures (20). However, the mechanisms involved in these activities of enamel extracts are not well understood.

Previous studies show that low-molecular-mass amelogenin from bovine dentin has chondrogenic activity (37), and recombinant rat low-molecular-mass amelogenins induce signaling effects for mesenchymal cells *in vivo* and *in vitro* (38). Nevertheless,

since normal periodontium is observed in the experience of amelogenin knockout mouse, it may be denied the amelogenin has osteoinductive ability (39).

The Emd-Gel appeared more effective on the induction of ALP activity in the HPDL cells than the EMD. This result was confirmed by the effect of the heat-treated EMD on the ALP activity. The EMD heat treated at 60°C, 80°C, and 100°C was over two-fold more effective than the EMD without heat treatment on the ALP activity. In addition, the Emd-Gel was 1.7-fold more effective than the EMD.

The Emd-Gel and EMD exhibited the same protein profile, which contained mainly amelogenin and its derivatives. The maximum difference between Emd-Gel and EMD occurs with or without the heat treatment. By the heat treatment, the proteinase activities were inactivated in the Emd-Gel. When the EMD was heated for 2 h at 60°C, 80°C, or 100°C, no different band between with heat treatment and without was detected on the SDS-PAGE. These results indicate that the inhibitor against the induction of ALP activity on the HPDL cells is contained in the EMD, and is inactivated by the heating process.

This is the first study to show that Emd-Gel inactivated proteinase activity and inhibitor. Clinical study has demonstrated that the Emd-Gel and EMD have comparable effect for the treatment of intrabony defects (24). Our *in vitro* study supports that Emd-Gel has greater bioactivity than the EMD, considering that the protein content of Emd-Gel was 1.5 times as much as that of EMD. Therefore, the good clinical outcome can be expected by Emd-Gel application.

Acknowledgements

We thank all the members of our laboratories, especially Dr Y. Yamakoshi, Dr M. Kanazashi, and Dr T. Karakida. This work was performed in Tsurumi University High Technology Research Center. This work was partially supported by funding from the Bio-Venture Project from the Japanese Ministry of Education, Culture, Sports, Science, and Technology.

References

1. Urist MR. Bone histogenesis and morphogenesis in implants of demineralized enamel and dentin. *J Oral Surg* 1971; **29:**88–102.
2. Iwata T, Morotome Y, Tanabe T, Fukae M, Ishikawa I, Oida S. Noggin blocks osteoinductive activity of porcine enamel extracts. *J Dent Res* 2002;**81:**387–391.
3. Hammarström L. Enamel matrix, cementum development and regeneration. *J Clin Periodontol* 1997;**24:**658–668.
4. Hammarström L, Heiji L, Gestrelius S. Periodontal regeneration in a buccal dehiscence model in monkeys after application of enamel matrix proteins. *J Clin Periodontol* 1997;**24:**669–677.
5. Heijl L. Periodontal regeneration with enamel matrix derivative in one human experimental defect. A case report. *J Clin Periodontol* 1997;**24:**693–696.
6. Boyan BD, Weesner TC, Lohmann CH *et al.* Porcine fetal enamel matrix derivative enhances bone formation induced by demineralized freeze dried bone allograft in vivo. *J Periodontol* 2000;**71:**1278–1286.
7. Gestrelius S, Andersson C, Lidström D, Hammarström L, Somerman MJ. *In vitro* studies on periodontal ligament cells and enamel matrix derivative. *J Clin Periodontol* 1997;**24:**685–692.
8. Schwartz Z, Carnes DL, Pulliam R *et al.* Porcine fetal enamel matrix derivative stimulates proliferation but not differentiation of pre-osteoblastic 2T9 cells, inhibits proliferation and stimulates differentiation of osteoblast-like MG63 cells, and increases proliferation and differentiation of normal human osteoblast NHOst cells. *J Periodontol* 2000;**71:**1287–1296.
9. Tokiyasu Y, Takata T, Saygin E, Somerman MJ. Enamel factors regulate expression of genes associated with cementoblasts. *J Periodontol* 2000;**71:**1829–1839.
10. Ohyama M, Suzuki N, Yamaguchi Y, Maeno M, Otsuka K, Ito K. Effect of enamel matrix derivative on the differentiation of C2C12 cells. *J Periodontol* 2002;**73:**543–550.
11. Yoneda S, Itoh D, Kuroda S *et al.* The effects of enamel matrix derivative (EMD) on osteoblastic cells in culture and bone regeneration in a rat skull defect. *J Periodont Res* 2003;**38:**333–342.
12. Hu C-C, Bartlett JD, Zhang CH, Qian Q, Ryu OH, Simmer JP. Cloning, cDNA sequence, and alternative splicing of porcine amelogenin mRNAs. *J Dent Res* 1996;**75:**1735–1741.
13. Hu C-C, Fukae M, Uchida T *et al.* Cloning and characterization of porcine enamelin mRNAs. *J Dent Res* 1997;**76:**1720–1729.
14. Fukae M, Tanabe T. Nonamelogenin components of porcine enamel in the protein fraction free from the enamel crystals. *Calcif Tissue Int* 1987;**40:**286–293.
15. Hu C-C, Fukae M. Uchida T et al. Cloning, cDNA/polypeptide sequences, and immunolocalization of porcine enamel sheath proteins. *J Dent Res* 1997;**76:**648–657.
16. Krebsbach PH, Lee SK, Matsuki Y, Kozack CA, Yamada K, Yamada Y. Full-length sequence, localization, and chromosomal mapping of ameloblastin. A novel tooth specific gene. *J Biol Chem* 1996;**271:**4431–4435.
17. Cerny R, Slaby I, Hammarstrom L, Wultz T. A novel gene expressed in rat ameloblasts codes for proteins with cell binding domains. *J Bone Miner Res* 1996;**11:**883–891.
18. Fukae M, Tanabe T, Uchida T *et al.* Enamelysin (Matrix Metalloproteinase-20): Localization in the developing tooth and effects of pH and calcium on amelogenin hydrolysis. *J Dent Res* 1998;**77:** 1580–1588.
19. Simmer JP, Fukae M, Tanabe T *et al.* Purification, characterization, and cloning of enamel matrix serine proteinase 1. *J Dent Res* 1998;**77:**377–386.
20. Kawase T, Okuda K, Momose M, Kato Y, Yoshie H, Burns DM. Enamel matrix derivative (EMDOGAIN®) rapidly stimulates phosphorylation of the MAP kinase family and nucler accumulation of smad2 in both oral epithelial and fibroblastic human cells. *J Periodont Res* 2001;**36:**367–376.
21. Kingsley DM. The TGF-beta superfamily: new members, new receptors, and new genetic tests of function in different organisms. *Genes Dev* 1994;**8:**133–146.
22. Urist MR. Bone: formation by autoinduction. *Science* 1965;**150:**893–899.
23. Yamaguchi A, Ishizuya T, Kintou N *et al.* Effects of BMP-2, BMP-4, and BMP-6 on osteoblastic differentiation of bone-marrow derived stromal cell lines, ST2 and MC3T3–G2/PA6. *Biochem Biophys Res Commun* 1996;**220:**366–371.
24. Bratthall G, Lindberg P, Havemose-Poulsen A *et al.* Comparison of ready-to-use Emdogain®-gel and Emdogain® in patients with chronic adult periodontitis. *J Clin Periodontol* 2001;**28:**923–929.
25. Gesterilius S, Andersson C, Johansson AC *et al.* Formulation of enamel matrix derivative for surface coating. *J Clin Periodontol* 1997;**24:**678–684.
26. Somerman MJ, Archer SY, Imm GR, Foster RA. A comparative study of human periodontal ligament cells and gingival fibroblasts in vitro. *J Dent Res* 1988;**67:**66–70.

27. Ogata Y, Niisato N, Sakurai T, Furuyama S, Sugiya H. Comparison of the characteristics of human periodontal ligament cells. *J Periodontol* 1995;**66:**1025–1031.
28. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;**227:**680–685.
29. Heussen C, Dowdle EB. Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. *Anal Biochem* 1980;**102:**196–202.
30. Yamakoshi Y, Tanabe T, Fukae M, Shimizu M. Porcine amelogenins. *Calcif Tissue Int* 1994;**54:**69–75.
31. Yukna RA, Melloning JT. Histologic evaluation of periodontal healing in human following regenerative therapy with enamel matrix derivative. A 10-case series. *J Periodontol* 2000;**71:**752–759.
32. Sculean A, Junker R, Donos N, Windisch P, Brecx M, Dunker N. Immunohistochemical evaluation of matrix molecules associated with wound healing following treatment with an enamel matrix protein derivative in humans. *Clin Oral Invest* 2003;**7:**167–174.
33. McCulloch CAG. Progenitor cell populations in the periodontal ligament of mice. *Anat Rec* 1985;**211:**258–262.
34. Roberts WE, Morey ER. Proliferation and differentiation sequence of osteoblast histogenesis under physiogical conditions in rat periodontal ligament. *Am J Anat* 1985;**174:**105–118.
35. Wang EA, Rosen V, Cordes P *et al.* Purification and characterization of other distinct bone-inducing factors. *Proc Natl Acad Sci USA* 1988;**85:**9484–9488.
36. Wozney JM, Rosen V, Celeste AJ *et al.* Novel regulators of bone formation: molecular clones and activities. *Science* 1988;**242:**1528–1534.
37. Nebgen DR, Inoue H, Sasaby B, Wei K, Ho CS, Veis A. Identification of the chondrogenic-inducing activity from bovine dentin (bCIA) as a low-molecular-mass amelogenin polypeptide. *J Dent Res* 1999;**78:**1484–1494.
38. Veis A, Tompkins K, Alvares K *et al.* Specific amelogenin gene splice products have signaling effects on cells in culture and in implants in vivo. *J Biol Chem* 2000;**275:**41263–41272.
39. Gibson CW, Yuan ZA, Hall B *et al.* Amelogenin-deficient mice display an amelogenesis imperfecta phenotype. *J Biol Chem* 2001;**24:**31871–31875.

This document is a scanned copy of a printed document. No warranty is given about the accuracy of the copy. Users should refer to the original published version of the material.