Porcine Enamel Protein Fractions Contain Transforming Growth Factor-β1

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Background: Enamel extracts are biologically active and capable of inducing osteogenesis and cementogenesis, but the specific molecules carrying these activities have not been ascertained. The purpose of this study was to identify osteogenic factors in porcine enamel extracts.

Methods: Enamel proteins were separated by size-exclusion chromatography into four fractions, which were tested for their osteogenic activity on osteoblast-like cells (ST2) and human periodontal ligament (HPDL) cells.

Results: Fraction 3 (Fr.3) and a transforming growth factor-beta 1 (TGF-β1) control reduced alkaline phosphatase (ALP) activity in ST2 but enhanced ALP activity in HPDL cells. The enhanced ALP activity was blocked by anti-TGF-β antibodies. Furthermore, using a dual-luciferase reporter assay, we demonstrated that Fr.3 can induce the promoter activity of the plasminogen activator inhibitor type 1 (PAI-1) gene.

Conclusion: These results show that the osteoinductive activity of enamel extracts on HPDL cells is mediated by TGF-β1.
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KEY WORDS

Periodontal ligament; transforming growth factor beta1.

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Proteins extracted from the immature enamel matrix of developing teeth possess important biologic activities, such as the induction of osteogenesis[1-3] and cementogenesis.[4] For example, it was shown in in vivo and in vitro systems that enamel matrix derivatives (EMDs) have cementum- and osteopromotive activities[5] and stimulate the proliferation and differentiation of osteoblastic cells.[6]

In developing dental enamel, there are five major extracellular matrix proteins: three structural proteins and two proteinases. The cDNAs encoding these proteins have been cloned from the developing tooth germs of various mammals, such as bovines, humans, pigs, rats, and mice. The three structural proteins are amelogenin,[7] enamelin,[8] and sheathlin,[9,10] also known as ameloblastin[11] or amelin.[12] The two proteinases are enamelysin[13,14] and kallikrein-4 (KLK4), which is also known as enamel matrix serine proteinase 1 (EMSP-1).[15] Ameloblastin and amelin, first cloned from rat-tooth specific cDNA libraries, are homologs of porcine sheathlin.

The periodontal regeneration activity of EMD is generally accepted. This activity stimulates bone formation[1] or periodontal regeneration,[4,16] but the specific molecules that mediate these activities and their mechanisms of action are not well understood. Although it is generally assumed that the major structural components of the developing enamel matrix are responsible for the observed osteoinductive

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properties, recently, the induction of alkaline phosphatase (ALP) activity in ST2 cells by fractionated porcine enamel extracts was largely blocked by noggin, a bone morphogenetic protein (BMP) inhibitor.[1] Furthermore, EMD‖ and transforming growth factor-beta (TGF-β) stimulate rapid translocation of Smad 2 into the nucleus of oral epithelial and fibroblastic cells.[17] Some BMPs, which belong to the TGF-β superfamily,[18] can induce osteogenesis in vivo[19] and osteogenic differentiation in vitro.[20] Thus, some evidence suggests that growth factors in developing dental enamel may mediate the tissue regeneration of EMDs. The purpose of this study was to investigate and identify the bioactive agents in fractionated enamel proteins.

MATERIALS AND METHODS

All experimental procedures involving the use of animals were reviewed and approved by the Institutional Animal Care Program at Tsurumi University.

Extraction and Fractionation of Enamel Proteins

Tooth germs of permanent molars were dissected from the fresh mandibles of 6-month-old pigs purchased from a slaughterhouse. Soft tissues were removed, and the mineralized portion of the developing teeth was rinsed in cold saline and wiped.¶ Secretory stage enamel was obtained by scraping the enamel surface with a curet.[21] Pooled enamel scrapings were suspended in 10 volumes of 0.05 M Sörensen buffer (pH 7.4) and homogenized by means of a homogenizer# for 30 seconds at half speed. The homogenate was centrifuged for 10 minutes at 10,000 × g. This procedure was repeated three times. The supernatants were combined as the neutral soluble fraction. The pellets were suspended in 10 volumes of 0.05 M carbonate-bicarbonate buffer (pH 10.8) and extracted in the same manner as with the neutral buffer. The carbonate buffer supernatants were collected as the alkaline soluble fraction.[22,23]

Gel Filtration Chromatography

The alkaline soluble fraction was applied to a column of sephadex G-100 (4 × 100 cm) equilibrated with 0.05 M carbonate-bicarbonate buffer (pH 10.8) and run at a flow rate of 15 ml/hour. The eluate was monitored at 280 nm and collected in four fractions. An aliquot of each fraction was de-salted on a PD-10 column** in 0.5 M acetic acid and lyophilized.

Acrylamide Gel Electrophoresis

Samples were resolved by 15% polyacrylamide slab gels containing 1% sodium dodecyl sulfate (SDS) as described by Laemmli[24] and stained with Coomassie brilliant blue (CBB) R-250.

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.[25] We selected one-cell type HPDL cells, which had a positive and stable ALP activity. These HPDL cells were used to determine osteoblastic differentiation. The cells were maintained in the α modification of Eagle's medium (α-MEM)†† containing 10% fetal bovine serum (FBS)‡‡ and 1% antibiotics (100 U/ml penicillin-G and 100 μg/ml streptomycin sulfate§§) at 37°C in a humidified 5% CO_2 atmosphere. The osteoblast-like cell ST2,‖‖ a mouse bone marrow stromal cell line, was cultured in the same condition.

Mitogenic Assay

The mitogenic activity of fractionated enamel protein samples were assayed using 3-(4,5)-dimethyl-2-thiazolyl-2,5-diphenyl-2H tetrazolium bromide (MTT)¶¶ according to the manufacturer's instructions. The HPDL cells were incubated in 100 μl growth medium at an initial density of 3×10^3 cells/well. After 24 hours of incubation, the α-MEM containing 1.0% FBS and 50 μg/ml of samples was then changed. After 96 hours, 10 μl MTT (5mg/ml) was added to each well, and the cells were incubated for 4 hours. The medium was discarded, and 100 μl 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.##

Enzyme Assay (ALP activity)

The HPDL cells were distributed in 96-well plates at a density of approximately 5×10^5 cells/well and incubated for 24 hours. The growth medium was changed to contain with or without 10 nM 1α,25-dihydroxyvitamin D_3*** and 50 μg/ml enamel protein fractions dissolved in ultrapure water. After 96 additional hours of incubation, the cells were washed once with phosphate buffered saline (PBS), and ALP activity was assayed 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 minutes at 37°C. Adding NaOH quenched the reaction, and the absorbance at 405 nm was read on a plate reader.

‖ Emdogain, Biora, Malmö, Sweden.
¶ Kimwipes, Kimberly-Clark, Roswell, GA.
# Polytron, Kinematica, Littau, Switzerland.
** Amersham-Pharmacia Biotech, Uppsala, Sweden.
†† Life Technologies, Grand Island, NY.
‡‡ Asahi Technoglass, Chiba, Japan.
§§ Gibco BRL, Grand Island, NY.
‖‖ Riken Cell Bank, Tsukuba, Japan.
¶¶ Sigma, St. Louis, MO.
Bio-Rad Model 450, Bio-Rad, Hercules, CA.
*** Calbiochem, La Jolla, CA.

The ST2 cells were spread on 96-well plates at a density of 1×10^6 cells/well and incubated for 24 hours. The growth medium was changed to contain with or without 100 nM all-trans retinoic acid[†††] and 50 μg/ml fractionated enamel protein samples dissolved in ultrapure water. After 96 additional hours of incubation, the ALP activity was determined as described above. These samples and exogenous growth factors (BMP 2:500 ng/ml; TGF-β 1:50 ng/ml) were tested for ALP activity.

Anti-TGF-β Antibody Binding Assay

HPDL cells were distributed in 96-well plates at a density of $\sim 5 \times 10^5$ cells/well, and the ST2 cells were spread on 96-well plates at a density of 1×10^6 cells/well and incubated for 24 hours. Before the medium was changed, various concentrations of anti-TGF-β antibody[‡‡‡] and Fraction 3 (Fr.3; 30 μg/ml) were mixed and incubated at room temperature for 1 hour in the growth medium. Seventy-two hours after the medium was changed, ALP activity was determined as described in Enzyme Assay.

Dual Luciferase Reporter Assay

Mv1Lu mink lung epithelial cells were obtained.[§§§] Mv1Lu cells were maintained in Dulbecco's modified Eagle's medium (DMEM)[‖‖‖] containing 10% FBS and 1% antibiotics at 37°C in a humidified 5% CO_2 atmosphere. Mv1Lu cells were seeded at 1.5×10^4 cells/well in 96-well plates and incubated for 24 hours. Plasminogen activator inhibitor type 1 (PAI-1) has been used as a marker for TGF-β–induced transcription[26] and the induction of the PAI-1 gene in Mv1Lu cells. The PAI-1 reporter gene was stimulated through TGF-β1 receptor-Smad (R-Smad), Smad 2, and Smad 3. TGF-β1 activity has been determined in Mv1Lu cells[27] using a luciferase reporter gene conjugated to a TGF-β–responsive PAI-1 promoter region (PAI-1-luc). The PAI-1-luc was transfected in Mv1Lu cells by Lipofectamine 2000.[¶¶¶] Mv1Lu cells were deprived of serum for 4 hours of transient transfection, and the culture medium was replaced with fresh medium containing 2.5% FBS containing enamel protein fractions (50 μg/ml) and cultured for an additional 24 hours. Luciferase reporter assay was performed according to the supplier's protocol,[###] and the luciferase activity was measured.[28****]

Mineralization Activity

The HPDL cells were plated in 24 well plates at an initial density of 3×10^4 cells/well. After 24 hours 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 and 25μg/ml of Fr.3. The medium was changed every 72 hours. Cells were maintained for 21 days and then the medium was discarded.

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

For measuring the calcium contents, the compartments of cells were dissolved by 0.5 N hydrochloric acid. The resulting solution was measured using a testing kit and following the manufacturer's protocol.[‡‡‡‡] The absorbance at 570 nm was read on a plate reader.

Statistical Analysis

All values are represented as means ± SE. Statistical significance was determined using an unpaired Student *t* test, and $P < 0.01$ was considered statistically significant.

RESULTS

Isolation of Porcine Enamel Proteins and Protein Profiles

Porcine enamel proteins were extracted under alkaline conditions and separated into four fractions (Fig. 1A). The SDS-polyacrylamide gel electrophoresis (PAGE) profiles corresponding to Fr.1 through Fr.4 are shown in Figure 1B. The elution of almost all enamel proteins on this system depended on their molecular size, except for the first eluted peak that was an aggregate containing low–molecular-weight proteins.[29] Fr.2 contains mainly 25-, 23-, and 20-kDa proteins, Fr.3 contains 20-, 13-, 6-kDa proteins, and Fr.4 contains 6-kDa proteins, all of which were determined to be amelogenins.

Fr.3 Induced ALP Activity of HPDL Cells

The growth-stimulative effect of Fr.1 through Fr.4 was examined by mitogenic assay. Each sample at concentrations of 50μg/ml did not show any growth-stimulative effect (data not shown). In HPDL cells, the ALP activity was enhanced by Fr.3 and TGF-β1 (Fig. 2A). This effect of Fr.3 with 1α,25-dihydroxyvitamin D_3 was more stable than without 1α,25-dihydroxyvitamin D_3. In contrast, BMP-2 and Fr.2 reduced ALP activity. On the other hand, Fr.2 and BMP-2 increased and Fr.3 and TGF-β1 decreased ALP activity in ST2 cells (Fig. 2B). This Fr.2 effect with all-trans retinoic acid was more stable than without all-trans retinoic acid. The influence of the cell stimulation by Fr.2 or

††† Sigma.
‡‡‡ R&D Systems, Minneapolis, MN.
§§§ Riken Cell Bank.
‖‖‖ Life Technologies.
¶¶¶ Invitrogen, Carlsbad, CA.
Dual-Luciferase Reporter Assay System, Promega, Madison, WI.
**** MiniLuminant LB 9506, Berthold, Bad Wildbad, Germany.
†††† Sigma.
‡‡‡‡ Calcium C-Test kit, WAKO, Osaka, Japan.

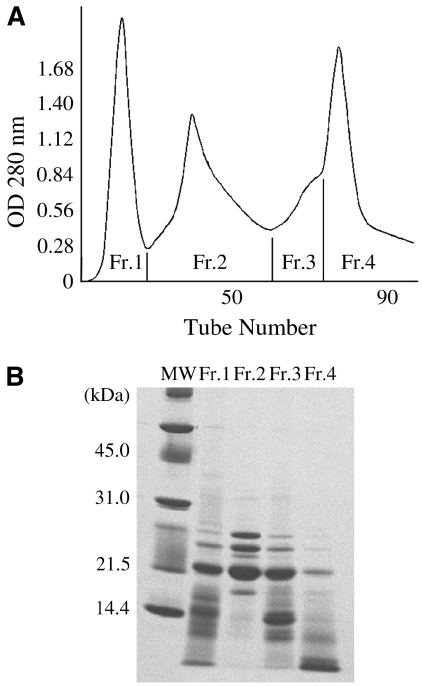

Figure 1.
Elution profile of fractionated porcine enamel proteins from the Sephadex G-100 column. ***A)*** *The chromatogram represents absorbance at 280 nm.* ***B)*** *SDS-PAGE profiles of fractions of Fr.1 through Fr.4 stained with CBB. Molecular weights (Bio-Rad Low Range Standards, Hercules, CA) are shown in left-hand margin.*

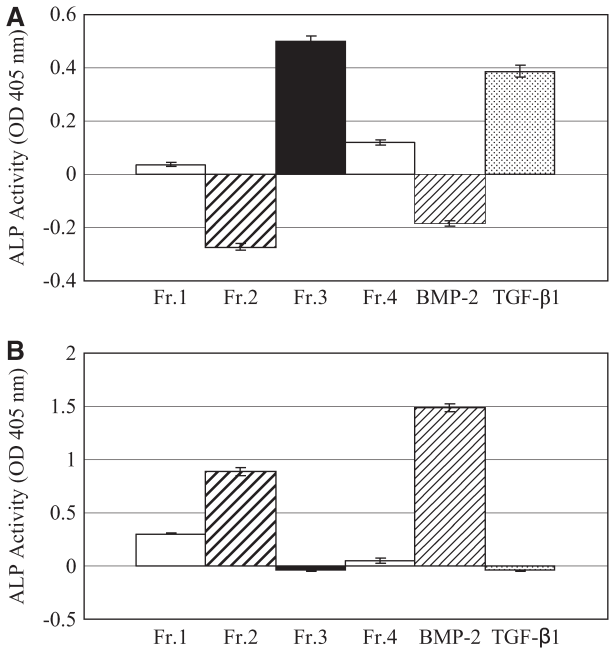

Figure 2.
Bars show ALP activities of HPDL cells and ST2 cells exposed four fractions. ***A)*** *The ALP activity of HPDL cells increased by Fr.3 (50 μg/ml) and TGF-β1 (50 ng/ml).* ***B)*** *The ALP activity of ST2 cells decreased by Fr.3 and TGF-β1. On the other hand, the ALP activity of ST2 cells increased Fr.2 (50 μg/ml) and BMP-2 (500 ng/ml). Data are means ± SE of three culture wells.*

Fr.3 was an opposite reaction with or without 1α,25-dihydroxyvitamin D_3 and all-trans retinoic acid in this assay system.

Anti-TGF-β Antibody Blocked ALP Activity of Fr.3 in a Dose-Dependent Manner

Anti-TGF-β antibody was incubated with Fr.3 and then added to HPDL or ST2 cells. The results showed that the ALP activity of the HPDL cells incubated by adding the Fr.3 was dose dependently decreased by the anti-TGF-β antibody (Fig. 3A) and was almost completely blocked at higher concentrations >1 μg/ml. On the other hand, the decrease of ALP activity was blocked by anti-TGF-β antibody in ST2 cells (Fig. 3B).

Luciferase Reporter Assays for Fractionated Porcine Enamel Proteins

The PAI-1 promoter activity using a dual-luciferase reporter assay, which was normally upregulated by TGF-β1 stimulation, was induced by Fr.3 (Fig. 4). Fr.1, Fr.2, and Fr.4 did not react with PAI-1 promoter activity.

Fr.3 Induced Mineralization Activity of HPDL Cells

When the compartment of HPDL cells was stained with alizarin red S at the end of the experimental period (days 21), many mineralized nodules were distinctly stained in the Fr.3 compared to the control. The calcium contents were also increased by Fr.3. The result shows that the calcium contents stimulated by the Fr.3 were almost two-fold higher than that resulting from the control (Fig. 5).

DISCUSSION

A mixture of porcine enamel proteins has been used clinically for the induction of cementogenesis along the tooth surface.[4] However, the mechanisms involved in these activities of enamel extracts are not well understood. It has been reported that EMD does not contain osteogenic factors,[6] and yet it has been demonstrated that porcine enamel extracts contain BMPs.[1]

After gel filtration of the alkaline soluble fraction by the sephadex G-100 column, Fr.3 enhanced ALP activity in HPDL cells. We also confirmed the osteoinductive activity by alizarin red staining and calcium-content assay. In HPDL cells, the osteoinductive activity was enhanced by Fr.3 and TGF-β1.[30] In addition, ALP activity was blocked in both cases by the anti-TGF-β antibody. These results indicate that Fr.3 contains TGF-βs that are antagonized by anti-TGF-β antibody. Furthermore, we showed the PAI-1 promoter activity, which was normally upregulated

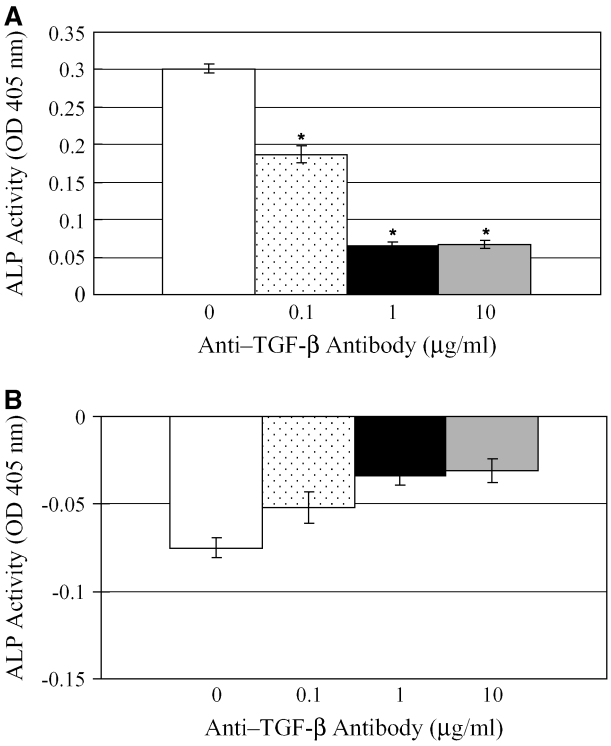

Figure 3.
The influence of anti-TGF-β antibody incubated with Fr.3 (30 μg/ml) of HPDL cells ***(A)*** *or ST2 cells* ***(B)****. Data are means ± SE of three culture wells. *Significantly different from culture without antibody at P <0.01.*

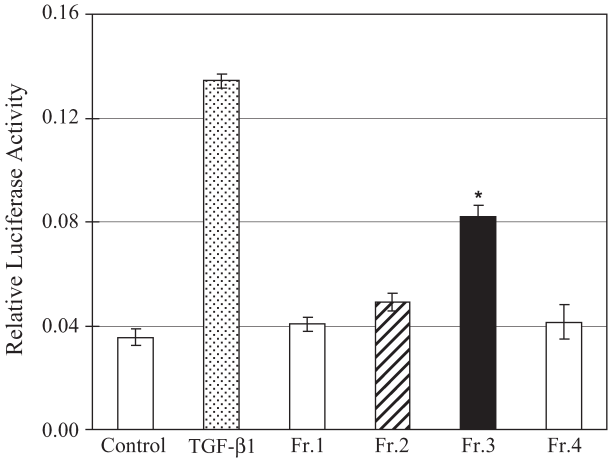

Figure 4.
*MV.1.Lu cells were transfected with the PAI-1 gene and treated with TGF-β1 (5 ng/ml) of Fr.1 through Fr.4 (50 μg/ml). The PAI-1 promoter activity of MV.1.Lu cells was induced by Fr.3. Data are means ± SE of four culture wells. *Significantly different from control at P <0.01.*

by TGF-β1 stimulation, was induced by Fr.3. We conclude that osteoinductive activities of fractionated enamel proteins on HPDL cells are mediated by TGF-β1. On the other hand, Fr.2 and BMP-2 reduced

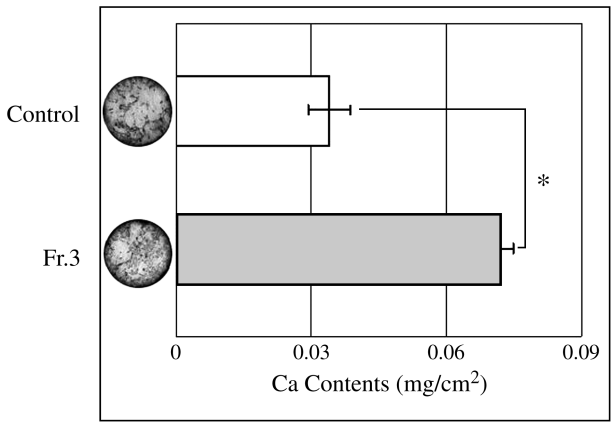

Figure 5.
*The mineralization activity of HPDL cells was induced by Fr.3. The mineralized nodules were stained with alizarin red S (left circles), and calcium contents (right bars) are shown. Data are means ± SE of three culture wells. *Significantly different from control at P <0.01 on calcium contents.*

ALP activity in HPDL cells. We confirmed the additional experiment with 10 nM 1α,25-dihydroxyvitamin D_3 in ST2 cells and 100 nM all-trans retinoic acid in HPDL cells. However, there was no influence in our result (data not shown). Furthermore, our findings support the previous in vitro study,[1] which reported that Fr.2 contains BMP-like growth factors.[1] These results suggest that the enamel matrix contains both BMP and TGF-β-like growth factors, and HPDL cells respond differently to BMP-2 and TGF-β1 stimulation.

Previously, Western blot analysis could not detect any bands corresponding to BMPs in porcine enamel extracts.[1] We tried Western blot analysis, but this technique may not be sufficiently sensitive to detect biologically relevant levels of these potent signaling molecules. Therefore, we performed a reverse transcription-polymerase chain reaction (RT-PCR) to confirm the expression of TGF-β1 mRNAs and TGF-β1–receptor mRNAs in porcine ameloblasts. Using specific primer sets based upon human and mouse mRNA sequences, we detected the mRNAs of TGF-β1 and the TGF-β1 receptor in a cDNA library of porcine secretory ameloblasts, as previously described.[31] Some reactions of Fr.3 and TGF-β1 on ALP activity are not enough to explain that the bioactivity of Fr.3 is related to TGF-β1. Consequently, enamel protein fractions were investigated using TGF-β1 reporter gene, high-detective TGF-β1 specific assay. We successfully detected the TGF-β1 activity of Fr.3.

CONCLUSIONS

To our knowledge, this is the first study to suggest that fractionated porcine enamel proteins contain both

BMPs and TGF-βs. Because the concentration is very low, the detection of BMPs and TGF-βs in enamel proteins is difficult. However, we successfully identified TGF-β activity in fractionated enamel proteins by using HPDL cell cultures. In this study, we showed grounds that EMD was useful in periodontal clinical procedures. Therefore, a good clinical outcome can be expected by EMD applications. We conclude that the enamel matrix of developing teeth contains TGF-β1, which may interact with BMPs and contribute to the differentiation of periodontal mesenchymal cells, and the osteoinductive activity of EMD on HPDL cells is mediated by TGF-β1.

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