J Periodont Res 2007; 42; 131–137

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2006.00926.x

Enamel matrix derivative stimulates chondrogenic differentiation of ATDC5 cells

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Narukawa M, Suzuki N, Takayama T, Shoji T, Otsuka K, Ito K. Enamel matrix derivative stimulates chondrogenic differentiation of ATDC5 cells. J Periodont Res 2007; 42: 131–137. © Blackwell Munksgaard 2006

Background and Objectives: Although enamel matrix derivative can promote chondrogenic differentiation of pluripotent mesenchymal precursor cells, the molecular mechanism that underlies this phenomenon is unclear. The purpose of this study was to determine the effect of enamel matrix derivative on chondrogenic differentiation. ATDC5 cells, which undergo a reproducible multistep chondrogenic differentiation, were cultured with or without enamel matrix derivative for up to 35 d.

Methods and Results: Cell proliferation and alkaline phosphatase activity increased markedly in cells cultured in the presence of enamel matrix derivative, compared with cells cultured in its absence. Deposition of Alcian blue-positive cartilage matrix and Alizarin red-positive mineralized nodules also increased significantly upon treatment with enamel matrix derivative. Expression of mRNAs encoding cartilage extracellular matrix proteins (type II collagen, type X collagen and aggrecan) and chondrogenic-related transcription factors (Sox9, Zfp60 and AJ18) were measured using the real-time polymerase chain reaction. Type II collagen, type X collagen and aggrecan mRNA expression increased markedly with enamel matrix derivative treatment. Transcription of Sox9, an important transcription factor that mediates chondrogenic differentiation, also increased with enamel matrix derivative treatment. The KRAB/C2H2 zinc-finger transcription factors, Zfp60 and AJ18, were transiently expressed in the prehypertrophic stage, and their expression increased with enamel matrix derivative treatment. In a western blot analysis with anti-insulin-like growth factor-I and anti-bone morphogenetic protein-6 immunoglobulin, bands corresponding to ≈ 14, ≈ 18 and ≈ 60 kDa were found in enamel matrix derivative.

Conclusion: Our study provides clear evidence that enamel matrix derivative promotes chondrogenic differentiation of ATDC5 cells.

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Key words: bone morphogenetic protein-6; chondroblasts; enamel matrix derivative; insulin-like growth factor-1

Accepted for publication June 8, 2006

One of the most important aspects of successful periodontal treatment is the regeneration of multiple periodontal tissues accompanying the newly formed cementum and alveolar bone (1,2). Enamel-related matrix proteins secreted by Hertwig's epithelial root sheath cells induce root formation and cementogenesis (3,4). In 1997, an enamel matrix derivative obtained from porcine embryonic enamel matrix extracts became commercially available. This product has been widely used in clinical settings to promote the formation of both cementum and alveolar bone (5). *In vitro* studies have demonstrated that enamel matrix derivative stimulates the proliferation of human periodontal ligament-derived cells, as well as protein synthesis and mineralization by these

cells (6). Enamel matrix derivative also stimulates the differentiation of primary osteoblasts and of osteoblastic cell lines (7,8).

We previously reported that enamel matrix derivative diverts the pluripotent mesenchymal cell line, C2C12, to an osteogenic and/or chondrogenic differentiation pathway (9). Recently, it has been demonstrated that enamel matrix derivative increases the expression of runt-related transcription factor-2 (Runx2) and induces the phosphorylation of mothers against decapentaplegic homologue 1 (Smad1) (10). These effects were proposed to be mediated by bone morphogenetic protein-2/4-like, or bone morphogenetic protein-7-like, molecules present in the enamel matrix derivative.

Although the enamel matrix derivative has proven to be a valuable material for clinical applications, such as the production of new cementum and alveolar bone, neither its effects on chondrogenic differentiation, nor the molecular components or mechanism responsible for these effects, have been clearly defined in C2C12 cells. In the course of chondrogenesis, at least two steps are regulated by local and systemic factors. These steps are (i) mesenchymal condensation, giving rise to proliferating chondrocytes, and (ii) differentiation of the proliferating chondrocytes to hypertrophic cells, followed by mineralization of the cartilage matrix (11).

In initial studies, enamel matrix derivative did not appear to contain any of the well-known growth factors, including basic fibroblast growth factor, insulin-like growth factor-I, platelet-derived growth factor-BB and transforming growth factor-β (6). However, recent studies have revealed that enamel matrix derivative contains molecules similar to bone sialoprotein (12), transforming growth factor-β (13) and bone morphogenetic protein (10). In the present study, we examined the effect of enamel matrix derivative on chondrogenic differentiation and sought to identify the enamel matrix derivative components involved in the chondrogenic differentiation of ATDC5 cells.

Material and methods

Cell culture

The chondrogenic cell line, ATDC5 (14), was obtained from the Riken Cell Bank (Tsukuba, Japan). The cells were maintained in a growth medium consisting of Dulbecco's modified Eagle's medium/Ham's F12 hybrid medium (Gibco Life Technologies, Rockville, MD, USA) containing 10% (v/v) fetal bovine serum (FBS; HyClone Laboratories, Logan, UT, USA), 1% (v/v) penicillin–streptomycin solution (50 U/ml penicillin and 50 μg/ml streptomycin; Sigma, St Louis, MO, USA), and 1% (v/v) of ITS, a mixture of insulin (10 μg/ml), transferrin (10 μg/ml) and Na-selenite (10 ng/ml) (Gibco Life Technologies). Cells were grown at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

To examine the effects of enamel matrix derivative on chondrogenic differentiation, cells were inoculated onto tissue culture plates at a density of 1×10^4 cells/cm^2 and incubated overnight. The growth medium was replaced with fresh medium containing 0, 10, 50 or 100 μg/ml of enamel matrix derivative (Emdogain; Biora AB, Malmö, Sweden) and was changed every second day. On day 28, the medium was removed and replaced with α-minimal essential medium containing 5% fetal bovine serum, 1% ITS and 50 mM β-glycerophosphate, and the CO_2 concentration was decreased to 3% to facilitate mineralization (15).

Cell proliferation

Cells were plated on 96-well microtiter plates, at a density of 1×10^4 cells/cm^2, and grown in the presence of 0, 10, 50 or 100 μg/ml of enamel matrix derivative for up to 35 d. The medium change ~ the medium was replaced with fresh medium containing 10% (v/v) of a colorimetric cell-counting kit reagent (Wako Pure Chemicals, Dosho-Machi Osaka, Japan), and incubation was continued for 2 h. Product formation was then assessed by the intensity of absorbance at 450 nm, as measured using a Titertek Multiscan Plus microtiter plate reader (Flow Laboratories, McLean, VA, USA). Relative cell numbers were calculated from the relative absorbance values by extrapolation from a standard curve.

Histochemical analysis

ATDC5 cells were plated onto 48-well microtiter plates at a density of 1×10^4 cells/cm^2 and cultured with 0, 10, 50 or 100 μg/ml of enamel matrix derivative for up to 35 d. Chondrogenic differentiation of ATDC5 cells was determined by staining of sulfated glycosaminoglycans with Alcian blue, as described previously (16). Briefly, cells were washed with phosphate-buffered saline (PBS), fixed with 4% (w/v) paraformaldehyde for 10 min, and stained with 0.5% (w/v) Alcian blue 8GX (Wako Fine Chemicals) in 0.1 M HCl overnight. The cells were washed with distilled water and examined by phase-contrast microscopy (Nikon, Tokyo, Japan). Alizarin red was used to assess mineralization. Cells were rinsed with PBS and then fixed with 2.5% (v/v) glutaraldehyde for 20 min before staining with 1% (w/v) Alizarin red S (Wako Fine Chemicals), as described previously (17). Cells were stained for 5 min and then destained with distilled water and examined by microscopy.

Alkaline phosphatase activity

Cells were plated in 96-well microtiter plates, at a density of 1×10^4 cells/cm^2, and cultured with 0, 10, 50 or 100 μg/ml of enamel matrix derivative for up to 35 d. Two-hundred microliters of enzyme reaction solution (8 mM *p*-nitrophenyl phosphate, 12 mM $MgCl_2$ and 0.1 mM $ZnCl_2$, in 0.1 M glycine-NaOH buffer, pH 10.5) was added to the cells in each well, and the plate was incubated for several minutes at 37°C. The enzyme reaction was terminated by the addition of 50 μl of 0.5 M NaOH. The amount of *p*-nitrophenol released by the enzyme reaction was determined by measuring the absorbance at 405 nm using a microtiter plate reader. One unit of alkaline phosphatase activity was defined as the amount of enzyme required to liberate 1.0 μmol of *p*-nitrophenol per minute.

Enzyme activity was reported as mU/10^4 cells.

Real-time polymerase chain reaction (real-time PCR)

Total RNA was isolated from cultured ATDC5 cells using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Aliquots containing equal amounts of mRNA were subjected to real-time PCR. First-strand cDNA synthesis was carried out using 1 μg of DNase-treated total RNA in 20 μl of a solution containing first-strand buffer, 50 ng of random primers, 10 mM dNTPs, 1 mM dithiothreitol and 0.5 U reverse transcriptase, at 42°C for 60 min.

The cDNA mixtures were diluted five-fold in sterile distilled water, and 2 μl aliquots were subjected to real-time PCR using SYBR Green I dye. The PCR was performed in 25 μl of a solution containing 1 × R-PR buffer, 1.5 mM dNTP mixture, 1 × SYBR Green I, 15 mM $MgCl_2$, 0.25 U ExTaq polymerase (real-time PCR version; TaKaRa, Tokyo, Japan), and 20 mM specific primers (sense and antisense), as shown in Table 1. Primers were designed using PRIMER3 software (Whitehead Institute for Biomedical Research, Cambridge, MA, USA). PCR was carried out in a thermal cycler (Smart Cycler, Cepheid, Sunnyvale, CA, USA), and the data were analyzed using the SMART CYCLER software 1.2d. The PCR conditions were 95°C for 3 s and 68°C for 20 s, for 40 cycles, and measurements were taken at the end of the annealing step at 68°C of each cycle.

PCR product specificity was verified by melting curve analysis between 68 and 94°C. All real-time PCR reactions were performed in triplicate, and the levels of mRNA expression were calculated and normalized to the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA at each time point.

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and western blotting

SDS–PAGE was carried out at 150 V for 60 min, in 5–20% gradient cross-linked polyacrylamide gels in a discontinuous Tris-glycine buffer system, as described by Laemmli (18). Immunotransfer was then carried out using a semidry transfer unit with a continuous buffer system, at 0.8 mA/cm^2 for 60–90 min. On completion of the transfer, the transfer membrane was blocked with 25% (v/v) Block Ace (Snow Brand Milk Products, Sapporo, Japan) at 4°C for 16 h. The membrane was then washed with Tris-buffered saline (TBS) containing Tween-20 (TBS-T).

For immunodetection, the blocked membrane was incubated at room temperature for 90 min with anti-insulin-like growth factor-I or anti-bone morphogenetic protein-6 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and then washed in TBS-T. The membrane was then incubated at room temperature for 60 min with a biotin-conjugated secondary antibody, washed with TBS-T and incubated for 20 min at room temperature with horseradish peroxidase-conjugated streptavidin. Immunoreactive proteins were visualized using a commercially available chemiluminescence kit (ECL Western blotting detection reagents; Amersham Biosciences, Bucks., UK) with exposure of the transfer membrane to X-ray film (Eastman Kodak, Rochester, NY, USA). As a positive control, recombinant human insulin-like growth factor-I (Invitrogen, Rockville, MD, USA) and recombinant human bone morphogenetic protein-6 (R & D Systems, Inc., Minneapolis, MN, USA) were used.

Statistical analyses

All data are represented as the mean ± standard deviation. Statistical significance was determined using Bonferroni's modification of the Student's *t*-test. The results were considered to be statistically significant at $p < 0.05$.

Results

Cell proliferation and histochemical analysis

When ATDC5 cells were cultured in the presence of 0–100 μg/ml of enamel matrix derivative for up to 35 d, cell proliferation was found to increase in the presence of enamel matrix derivative, in a dose-dependent manner (Fig. 1). Cartilage matrix and mineralized nodule formation were also measured (Fig. 2A), and enamel matrix derivative treatment was found to markedly increase Alcian blue-positive cartilage matrix deposition in a dose- and time-dependent manner. Mineralized nodules were visible to the naked eye on day 35. The effects of different doses of enamel matrix derivative on Alizarin red S staining of ATDC5 cells over time are shown in Fig. 2B. Enamel matrix derivative treatment

Table 1. Real-time polymerase chain reaction primers used in the experiments

Target	Forward primer	Reverse primer	Genbank acc. no.
Type II collagen	5′-ATGACAATCTGGCTCCCAAGACTGC-3′	5′-GACCGGCCCTATGTCCACACCGAAT-3′	NM_001844
Type X collagen	5′-AAAGCTTACCCAGCAGTAGG-3′	5′-ACGTACTCAGAGGAGTAGAG-3′	X67348
Aggrecan	5′-CTACGACGCCATCTGCTACA-3′	5′-ACGAGGTCCTCATCGGTGAA-3′	X80278
Sox9	5′-ATCTGAAGAAGGAGAGCGAG-3′	5′-TCAGAAGTCTCCAGAGCTTG-3′	AF421878
Zfp60	5′-CGTCTTACTAGAGCCGGAGAAA-3′	5′-ACTTACAACCAAAGCACTTCCC-3′	U48721
AJ18	5′-CCCCAAGGAAGTCACCAGT-3′	5′-CTTTCTATGGGATCGGTCTCTT-3′	AF32874
GAPDH	5′-GAGTCAACGGATTTGGACGT-3′	5′-GACAAGCTTCCCGTTCTCAG-3′	NG_003018

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

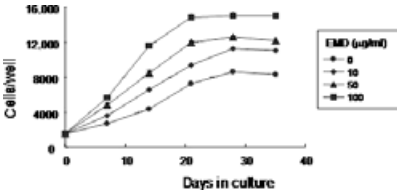

Fig. 1. Effect of enamel matrix derivative treatment on cell proliferation. ATDC5 cells were cultured with 0, 10, 50 or 100 μg/ml of enamel matrix derivative, and cell numbers were determined on days 7, 14, 21, 28 and 35 of culture. EMD, enamel matrix derivative.

clearly increased the resulting intensity of Alizarin red S staining, compared with cultures not exposed to enamel matrix derivative.

Alkaline phosphatase activity

ATDC5 cells were cultured in the presence of 0–100 μg/ml of enamel matrix derivative for up to 35 d, and alkaline phosphatase activity was measured (Fig. 3). Enamel matrix derivative was found to increase alkaline phosphatase activity significantly, in a dose-dependent manner, after 14 d of culture.

Chondrogenesis-related gene expression

Expression of mRNAs for chondrogenesis-related proteins (type II collagen, type X collagen, aggrecan, Sox9, Zfp60 and AJ18) was determined by real-time PCR analysis of ATDC5 cells cultured in the presence of 0–100 μg/ml of enamel matrix derivative for up to 35 d. Expression of type II collagen mRNA increased gradually to day 21 and then decreased on day 28 in the control culture. Enamel matrix derivative treatment caused expression of type II collagen mRNA to be significantly higher at 21 d than in its absence (Fig. 4A). Expression of type X collagen (Fig. 4B) and aggrecan (Fig. 4C) mRNAs increased gradually with time to day 35, both in the absence and presence of enamel matrix derivative. At 21 d, expression of these mRNAs was significantly higher in the presence of high concentrations of enamel matrix derivative (50 or 100 μg/ml) compared with no enamel matrix derivative. In both control and enamel matrix derivative-treated cells, Sox9 mRNA expression increased gradually to day 14, and decreased from day 28 onwards. High concentrations of enamel matrix derivative dramatically increased Sox9 mRNA expression from days 14–28 (Fig. 4D). On day 21 of culture, Zfp60 mRNA expression was significantly higher in cells grown in the presence of high concentrations of enamel matrix derivative than in those grown in its absence (Fig. 4E). In the absence of enamel matrix derivative, AJ18 mRNA expression did not change markedly over 35 d of culture. High concentrations of enamel matrix derivative caused transient expression of AJ18 mRNA, which reached a maximum level on day 21 (Fig. 4F).

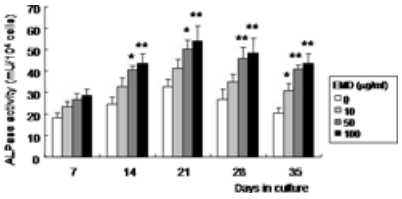

Fig. 3. Effect of enamel matrix derivative treatment on alkaline phosphatase activity. ATDC5 cells were cultured with 0, 10, 50 or 100 μg/ml of enamel matrix derivative, and alkaline phosphatase activity was determined on days 7, 14, 21, 28 and 35 of culture. The data shown are the mean ± standard deviation for three separate experiments; $*p < 0.05$, $**p < 0.01$, enamel matrix derivative-treated vs. control. ALPase, alkaline phosphatase; EMD, enamel matrix derivative.

Insulin-like growth factor-I-like and bone morphogenetic protein-6-like molecules in enamel matrix derivative

In western blot analysis, immunoreactive bands running at ≈14, ≈18 and ≈60 kDa were generated in response to anti-insulin-like growth factor-I and anti-bone morphogenetic protein-6 immunoglobulins, respectively, as shown in Fig. 5. This result indicates that enamel matrix derivative contains both insulin-like growth factor-I- and bone morphogenetic protein-6-like molecules.

Discussion

Although numerous studies have reported that enamel matrix derivative enhances osteoblastic differentiation, the precise mechanisms by which it controls chondrogenic differentiation remain unknown. Enamel matrix derivative was previously reported to cause pluripotent mesenchymal C2C12 cells to switch differentiation pathways to that of the osteoblast and/or chondroblast lineage, and it was considered that these effects might be mediated by bone morphogenetic protein-like mol-

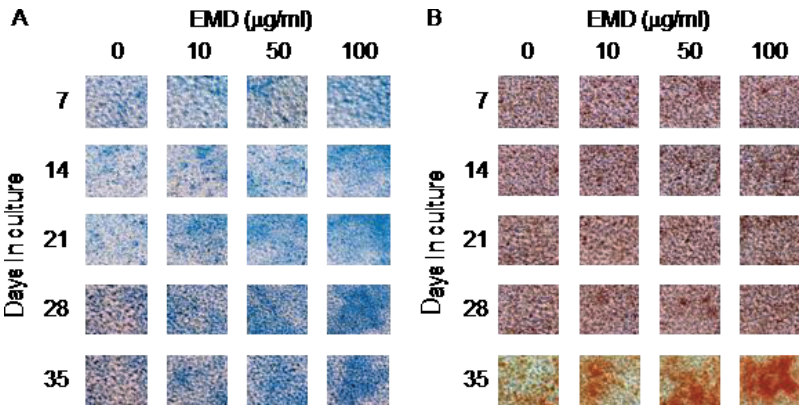

Fig. 2. Effect of enamel matrix derivative treatment on cartilage matrix and mineralized nodules formation. ATDC5 cells were cultured with 0, 10, 50 or 100 μg/ml of enamel matrix derivative, and formation of cartilage matrix was determined by staining with Alcian blue (A) and Alizarin Red S (B) on days 7, 14, 21, 28 and 35 of culture. (Original magnification ×100.) EMD, enamel matrix derivative.

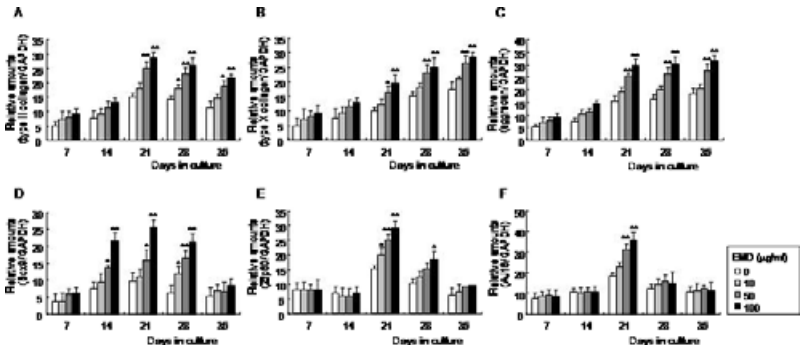

Fig. 4. Effect of enamel matrix derivative treatment on chondrogenesis-related gene expression. ATDC5 cells were cultured with 0, 10, 50 or 100 μg/ml of enamel matrix derivative for up to 35 d. On days 7, 14, 21, 28 and 35 of culture, expression of type II collagen (A), type X collagen (B), aggrecan (C), Sox9 (D), Zfp60 (E) and AJ18 (F) mRNA was determined using real-time polymerase chain reaction, as indicated. The data shown are the mean ± standard deviation for three separate experiments; $*p < 0.05$, $**p < 0.01$, enamel matrix derivative-treated vs. control. EMD, enamel matrix derivative; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

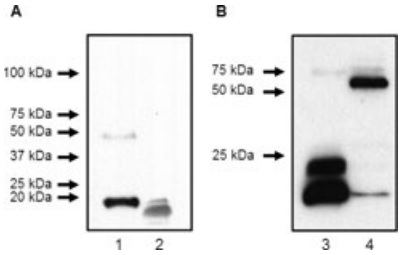

Fig. 5. Western blot of enamel matrix derivative using anti-insulin-like growth factor-I (A) and anti-bone morphogenetic protein-6 (B) immunoglobulin. Lane 1, recombinant human insulin-like growth factor-I; lanes 2 and 4, enamel matrix derivative; and lane 3, bone morphogenetic protein-6.

ecules (9,10). In the present study, we examined the effect of enamel matrix derivative on chondrogenic differentiation.

Initially, we examined the effect of enamel matrix derivative on cell proliferation. Our results indicated that enamel matrix derivative substantially increases cell proliferation in a dose-dependent manner. Because we previously demonstrated that enamel matrix derivative contains bone morphogenetic protein-2/4-like and/or bone morphogenetic protein-7-like molecules, and that these molecules affect mesenchymal cell differentiation (9,10), we surmised that these molecules were also responsible for the proliferative effect of enamel matrix derivative. Although insulin-like growth factor-I and basic fibroblast growth factor are reported to stimulate the proliferation of ATDC5 cells, bone morphogenetic protein-2 has little effect on cell proliferation (19).

Using immunoassays, Gestrelius *et al.* (6) were unable to detect the growth factors, calbindin D, granulocyte–macrophage colony-stimulating factor, epidermal growth factor, fibronectin, basic fibroblast growth factor, insulin-like growth factor-1, interleukin-1β, platelet-derived growth factor-BB, tumor necrosis factor, or transforming growth factor-β, in commercially available enamel matrix derivative. However, in more recent studies, molecules similar to bone sialoprotein (12), transforming growth factor-β (13) and bone morphogenetic protein (10) were detected in commercial enamel matrix derivative. Therefore, we thought that enamel matrix derivative might also contain other molecules that promote ATDC5 cell proliferation.

In an *in vitro* study, ATDC5 cells were shown to differentiate into proliferating chondrocytes through a cellular condensation process followed by cellular hypertrophy and mineralization (15). In the presence of enamel matrix derivative, chondrogenic differentiation was initiated, forming Alcian blue-positive cartilage nodules through a cellular condensation process that gives rise to proliferating chondrocytes by day 35. A reduction in CO_2 levels and the addition of β-glycerophosphate to the culture greatly facilitated progressive mineralization, and this effect was significantly enhanced by enamel matrix derivative. The present histochemical findings strongly suggest that enamel matrix derivative stimulates not only chondrocyte hypertrophy but also mineralization *in vitro*. Although the major of maxilla and mandibular are formed by intramembranous ossification, it was reported that during endochondoral bone formation, the hypertrophic chondrocytes act as an essential interface between cartilage and bone by facilitating transition from cartilage to bone and coupling chondrogenesis to osteogenesis and also angiogenesis (20). Therefore, enamel matrix derivative might be a useful material, in part, by stimulating periodontal regeneration by chondrogenesis.

Alkaline phosphatase, which hydrolyzes ester bonds of organic phosphate compounds under alkaline conditions, is considered to be a marker of chondrocyte differentiation; its activity appears to increase during chondrocyte hypertrophy (21,22). In this report, enamel matrix derivative was found to increase alkaline phosphatase activity significantly, in a dose-dependent manner. High levels of alkaline phosphatase activity are normally associated with hypertrophic chondrocytes and matrix vesicles, which accumulate in the extracellular matrix (23,24). These results strongly suggest that enamel matrix derivative increases chondrogenic differentiation.

On the other hand, enamel matrix derivative was reported to exert an inhibitory effect on alkaline phosphatase activity in chondrocytes (25). Whether this discrepancy arises from differences in cell types, culture conditions, methods, timing of enamel matrix derivative addition to culture, or some other parameter, is unknown.

Enamel matrix derivative treatment caused a significant increase in the expression of mRNAs encoding type II collagen and aggrecan, a marker of

prehypertrophic chondrocytes (26,27). Enamel matrix derivative treatment also caused an increased expression of mRNA encoding type X collagen, a marker of fully differentiated chondrocytes (28). These results suggest that enamel matrix derivative promotes not only hypertrophic differentiation, but also terminal differentiation. Enamel matrix derivative treatment also increased mRNA expression for Sox9, an important transcription factor that mediates chondrogenic differentiation (29,30). It is thus conceivable that enamel matrix derivative promotes chondrogenic differentiation by increasing Sox9 expression.

The KRAB/C2H2-type zinc finger transcription factors constitute the largest transcription factor family, which regulates many fundamental cellular processes, including cell differentiation and organ development (31). Zfp60 mRNA was found to be transiently expressed, coincident with chondrocyte maturation. As overexpression of Zfp60 decreases chondrogenic differentiation, Zfp60 may regulate cellular hypertrophy (32). AJ18, the first KRAB/C2H2 zinc finger protein reported to be expressed in developing bone (33), is also highly expressed in developing cartilage, where it may regulate osteoblastic and/or chondroblastic differentiation by affecting Runx2 activity (34). Because one or more of the aforementioned transcription factors could be responsible for the effect of enamel matrix derivative, we determined the effect of enamel matrix derivative treatment on their expression. Zfp60 was transiently expressed in the prehypertrophic stage, and its expression was increased by enamel matrix derivative treatment. The expression pattern of AJ18 in chondrogenic differentiation was similar to that of Zfp60, and AJ18 expression was also increased by enamel matrix derivative treatment. Thus, we conclude that enamel matrix derivative may regulate the amount of space that becomes filled by bone marrow through modulation of Zfp60 and AJ18 expression.

In light of the above findings, we assumed that the ability of enamel matrix derivative to promote chondrogenic differentiation was mediated by insulin-like growth factor-I-like and/or bone morphogenetic protein-6-like molecules present in enamel matrix derivative. Insulin-like growth factor-I is a well-documented stimulator of chondrocyte proliferation and differentiation (35–38). Moreover, bone morphogenetic protein-6, but not bone morphogenetic protein-2, was recently reported to promote osteogenic/chondrogenic differentiation strongly (39,40).

In an immunoblot experiment, we found that anti-insulin-like growth factor-I and anti-bone morphogenetic protein-6 immunoglobulins cross-reacted strongly with two enamel matrix derivative proteins with apparent molecular weights of $\approx$14, $\approx$18 and $\approx$60 kDa. As insulin-like growth factor-I and bone morphogenetic protein-6 migrate with apparent molecular weights of 7–10, $\approx$18 and $\approx$60 kDa, these results constitute the first evidence that enamel matrix derivative contains $\approx$14-kDa insulin-like growth factor-I-like protein and $\approx$18/$\approx$60-kDa bone morphogenetic protein-6-like proteins. Gestrelius *et al*. failed to detect insulin-like growth factor-I-like molecules in enamel matrix derivative by enzyme-linked immunosorbent assay (6). It is not clear whether this discrepancy is caused by differences in the assay procedures or is a result of other unknown parameters. Any apparent size discrepancies are readily explained by the fact that the enamel matrix derivative used in the present study was prepared from fetal porcine tooth germs, not from bone cells, and could thus contain different isoforms (41–44). We showed that enamel matrix derivative contains insulin-like growth factor-I- and bone morphogenetic protein-6-like molecules in this study, and we also previously demonstrated that bone morphogenetic protein-2-, -4-, and -7-like molecules might promote chondrogenic differentiation in enamel matrix derivative (10).

In conclusion, the ability of enamel matrix derivative to promote chondrogenic differentiation may be mediated, in part, by insulin-like growth factor-I-like and/or bone morphogenetic protein-like molecules, suggesting that enamel matrix derivative may be useful as a reagent for cartilage regeneration.

Acknowledgements

This study was supported by a multidisciplinary research project grant from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, by a grant from the Dental Research Center at the Nihon University School of Dentistry, and by the Sato Fund of the Nihon University School of Dentistry. The Promotion and Mutual Aid Corporation for Private Schools of Japan also supported this study.

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