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# Inhibitory effect of enamel matrix derivative on osteoblastic differentiation of rat calvaria cells in culture

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*Background and Objective:* The effect of enamel matrix derivative (EMD) on bone differentiation remains unclear. Transforming growth factor β1 (TGF-β1) is reported to be contained in EMD. The aim of this study was to clarify the effect of EMD on osteoblastic cell differentiation and the possible role of TGF-β1.

*Material and Methods:* Fetal rat carvarial cells were treated with 10, 50 or 100 μg/ml EMD for 5–17 days. Alkaline phosphatase (ALP) activity and bone nodule formation were measured, and mRNA expressions of bone matrix proteins and core binding factor were analysed.

*Results:* Enamel matrix derivative inhibited ALP activity from the early stage of culture (29–44% inhibition) on days 5 and 10 and decreased bone nodule formation by 37–67% on day 17. These effects of EMD were concentration dependent. Enamel matrix derivative inhibited mRNA expression of osteocalcin and core binding factor. A high level of the active form of TGF-β1 protein was detected in the conditioned medium treated with 100 μg/ml EMD. Treatment with TGF-β1 antibody partly restored the inhibitory effect of EMD on ALP activity.

*Conclusion:* Enamel matrix derivative inhibited the osteoblastic differentiation of rat carvarial cells and this was partly mediated by an increase in the activated form of TGF-β1, suggesting that EMD may function initially to inhibit osteoblastic differentiation to allow a predominant formation of other periodontal tissues.

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Enamel matrix proteins are secreted by Hertwigh's epithelial root sheath and play a role in the attachment of periodontal apparatus such as cementum, periodontal ligament and alveolar bone during root development (1). Enamel matrix derivative (EMD) is a product purified from porcine enamel matrix protein extracts, and amelogenin is a major component (> 90%). It is reported that EMD induces the formation of acellular cementum on denuded root surfaces of monkey and human teeth, suggesting that EMD has the potential to stimulate periodontal tissue regeneration (2). Many clinical studies and animal experiments have demonstrated that EMD induced the formation of new layers of acellular cementum, periodontal ligament (PDL) and alveolar bone, and that clinical parameters such as probing depth and attachment level were markedly improved by application of EMD (3–10). Although it is clear that EMD is effective in the recovery of periodontal tissue defects, the regenerative mechanism of EMD in periodontal tissues has not been precisely elucidated.

*In vitro* studies have shown that EMD stimulates proliferation, cell spreading, alkaline phosphate (ALP)

activity and bone nodule (BN) formation in cultured periodontal ligament cells (11–15). These data are in agreement with the *in vivo* results and clinical studies previously reported. The effect of EMD on the proliferation and differentiation of osteoblastic cells depends on cell species and culture conditions. Enamel matrix derivative stimulated the proliferation of the murine preosteoblast cell line MC3T3-E1 (16) but did not affect 2T9 cells (17). Enamel matrix derivative inhibited proliferation and stimulated differentiation in the human osteoblast-like osteosarcoma cell line MG63 (17). In normal human osteoblast NHOst cells, EMD increased proliferation and differentiation (17). In 2T9 cells, EMD did not stimulate cell proliferation when the culture dish surface was coated with EMD prior to adding the cells (17).

In this study, we used fetal rat calvaria (RC) cells, which contain many undifferentiated mesenchymal cells and differentiate into osteoblastic cells and then form BNs *in vitro* (18). Since RC cells are primary osteoblastic cells reflecting the physiological condition, this culture system is useful to elucidate the effect of EMD on osteoblasts. The purpose of this study was to investigate the effect of EMD on RC cell differentiation by determining ALP activity, BN formation and mRNA expressions of bone matrix protein, osteopontin, osteocalcin and core binding factor (Cbfa1; osteoblast-specific transcription factor associated with bone growth).

Also, it was recently reported that EMD stimulated the proliferation of gingival fibroblasts and that this stimulatory effect was induced by transforming growth factor β1 (TGF-β1)-like molecules, present in EMD (19). Since TGF-β1 showed a multipotential action on growing cells, the present study focused on the association of TGF-β1 with the effect of EMD on RC cells.

## Material and methods

### Rat calvaria cell culture

Rat calvaria cells were prepared from calvariae of 21-day-old fetal Wistar rats (Charles River Laboratories, Yokohama, Japan) as described by Bellows *et al.* (18). Briefly, cells isolated by sequential digestion with an enzyme mixture containing collagenase were cultured in α Eagle's minimal essential medium (α-MEM; ICN Biomedical. Inc., Aurora, OH, USA) containing 10% fetal bovine serum (FBS; Lenexa, KA, USA) and antibiotics. After 24 h, RC cells were trypsinized and seeded at a density of 3300 cells/cm$^2$ into α-MEM containing 10% FBS, 50 μg/ml ascorbic acid, 2 mM β-glycerophosphate. After 24 h, media were replaced, with or without various concentrations of EMD gel (Biora, Malmö, Sweden; 10, 50 or 100 μg/ml). In the control medium, propylene glycol alginate (PGA, Biora) was diluted as an EMD solvent. The total amount of PGA was equally adjusted in the culture medium (1/100). In some experiments, RC cells (3300 cells/cm$^2$) were cultured with or without 1 ng/ml TGF-β1 (Boeringer Manheim, Manheim, Germany), and anti-TGF-β1 antibody (catalogue no. 41100, Genzyme-Techne, MA, USA; 0.2 μg/ml) was added for 5 or 17 days.

### Preparation of TGF-β1

Human recombinant TGF-β1 was dissolved at a concentration of 1 μg/ml in sterile phosphate-buffered saline (PBS; Nissui Pharmaceutical, Tokyo, Japan) as recommended by the manufacturer. Anti-TGF-β1 antibody for neutralization was prepared according to the method of Kawase *et al.* (20). From

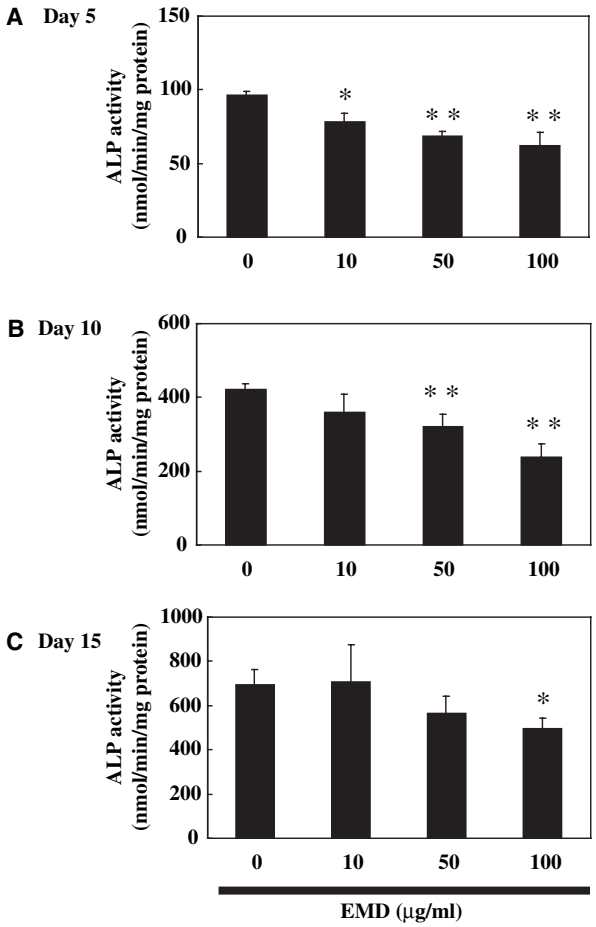


*Fig. 1.* Effect of EMD on ALP activity in RC cells. RC cells were cultured with EMD (10, 50 or 100 μg/ml) for 5–15 days. ALP activity in cell fractions was determined as described in Material and methods (A, day 5; B, day 10; and C, day 15). Values are means ± SE of triplicate samples in three separate experiments. *$P < 0.05$, **$P < 0.01$, EMD vs. control.

the manufacturer's protocol, we confirmed that this antibody showed a cross-activity with rat, porcine and bovine TGF-β1.

### Determination of ALP activity and number of bone nodules

To assay ALP activity, cells were scraped into 50 mM Tris–HCl buffer (pH 7.4), sonicated, and centrifuged at 2000*g* for 10 min at 4°C. The enzyme activity in the supernatant was determined using *p*-nitrophenyl phosphate as the substrate according to the method of Lowly *et al*. (21).

To assay BN formation, cells were washed in PBS, fixed with 10% neutral-buffered formation and stained *in situ* by the von Kossa technique (18). The number of BN stained as dark dots was determined using computer software (NIH image analyzer version 1.62) as reported previously (22).

### Northern blotting

Total cellular RNA from RC cells was extracted with acid guanidinium thiocyanate–phenol–chloroform (22) using an RNA isolation kit (Stratagene, La Jolla, CA, USA). Total RNA (10 μg) was resolved by electrophoresis in 1% agarose gels containing 0.82 M formaldehyde and transferred to a positively charged membrane (Hybond-N + nylon membrane, Amersham Pharmacia Biotech, Little Chalfont, UK). The probes for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were prepared by digesting pHcGAP with *Pst*I/BgII. Plasmids containing cDNAs of mouse osteopontin and rat osteocalcin were supplied by Dr D. T. Denhardt (Rugers University, Piscataway, NJ, USA) and Dr J. M. Wozney (Genetics Institute, Cambridge, MA, USA), respectively. The cDNA probes were labelled with [α-$^{32}$P]-dCTP (Amersham) using a random-primer DNA-labelling kit (Amersham). The membrane was prehybridized at 42°C in 50% formamide, 5 × SSPE, 5 × Denhardt's solution, 0.5% sodium dodecyl sulphate and 200 μg/ml salmon sperm DNA, and then hybridized at 42°C overnight in the same solution with $^{32}$P-labelled probes. The membrane was exposed to X-ray film (Amersham) with an intensifying screen for 6 h at −70°C. The hybridization signals were quantified using an imaging analyser (Fujix Bioimaging Analyzer; model BAS-2000II, Fuji Photo Film Co., Tokyo, Japan). The levels of several mRNAs were normalized to that of GAPDH mRNA.

### Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA (1 μg) was digested with 1 IU/μl AMV reverse transcriptase using cDNA synthesis kit (Life Sciences, Inc., St Petersburg, FL, USA) for 1 h at 42°C. The RT products were mixed with PCR buffer (100 μl) containing 150 nM of the 5′- and 3′-primers of Cbfa1 and TGF-β1 and 2.5 U Taq DNA polymerase and amplified for 30 or 35 cycles at 94°C for 1 min. The PCR products were resolved by electrophoresis in 2% agarose gels and stained in Tris-acetate, EDTA buffer (TAE; Wako Pure Chemical Industries, Osaka, Japan) containing 1 μg/ml ethidium bromide (Sigma-Aldrich, Tokyo, Japan), and then analysed using a fluorescence detector and its software (Epi-Light UV FA 500 and Luminous Imager version 2, AISiN Co., Tokyo, Japan). The primers for Cbfa1 were 5′-ACCTCTGACTTCTGCCTCTG-3′ and 5′-CGCCAAACAGACTCATCCAT-3′. The primers for TGF-β1 were 5′-CACCCCAAAGCTGGGGGGCA-3′ and 5′-GGGGTTCGCGCTCTCCGAAG-3′. The levels that represent the relative expression of mRNA by several PCR products were normalized to that of the GAPDH PCR product.

### Measurement of TGF-β1 in culture media

Rat calvaria cells were seeded in 96-well culture plates at a density 3300 cells/cm$^2$. After the treatment with EMD (10, 50 or 100 μg/ml) for 5, 10 and 15 days, the amounts of TGF-β1 in the culture media were determined using an ELISA kit for rat TGF-β1 (Promega, Madison, WL, USA). Although TGF-β1 is generally processed from a latent form, the bioactive form is immunoreactive and detectable with the anti-TGF-β1 anti-

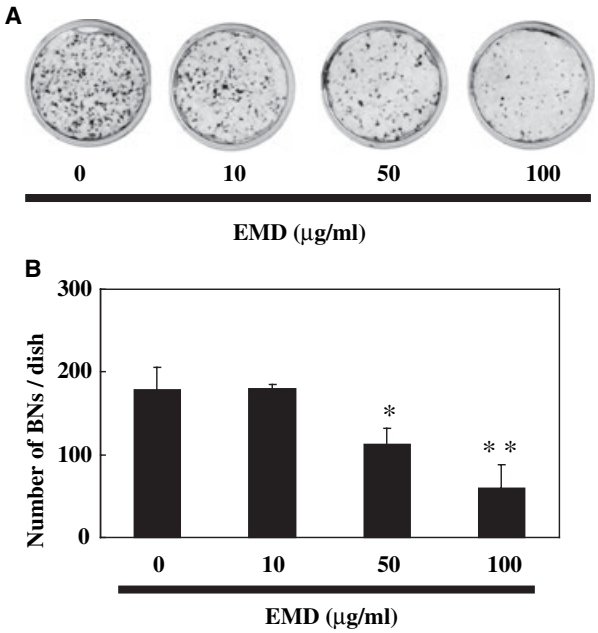


*Fig. 2.* Effect of EMD on BN formation in RC cells. (A) RC cells were cultured with EMD (10, 50 or 100 μg/ml) for 15 days and stained by the von Kossa method. (B) Numbers of stained BNs were determined by computer analysis. Values are means ± SE of triplicate samples in three separate experiments. $*P < 0.05$, $**P < 0.01$, EMD vs. control.

body used in this experiment. In order to determine the total TGF-β1, comprising the latent and bioactive forms, we quantified the samples using 1 N hydrochloride (HCl) according to the ELISA kit manufacturer's protocol.

### Statistical analysis

Data were statistically analysed using the variance (ANOVA) and the multiple comparison procedure of Scheffe. Differences at $P < 0.05$ were considered significant.

## Results

### Enamel matrix derivative inhibits ALP activity and BN formation in RC cells

Rat calvaria cells were cultured with 10, 50 or 100 μg/ml EMD for 17 days. As shown in Fig. 1, EMD significantly inhibited ALP activity in a concentration-dependent manner. Treatment with 10, 50 or 100 μg/ml of EMD decreased ALP activity to 64–83% of control values on day 5 (Fig. 1A), demonstrating inhibition from an early stage of culture. As shown in Fig. 1(B,C), this inhibition continued on day 10 (56–86% of the control values) and on day 15 (71% of the control values). Figure 2(A) shows culture dishes stained by the Von Kossa technique. On day 17, the intensity of mineralized BNs was weaker in EMD-treated dishes than in the control dishes. When the BNs were measured by computer analysis, EMD significantly decreased the number of BNs in a concentration-dependent manner, showing 63 and 33% of the control values at 50 and 100 μg/ml EMD, respectively (Fig. 2B). Thus, inhibitory effects of EMD were observed not only on ALP activity but also on BN formation.

### Enamel matrix derivative stimulates the mRNA expression of osteopontin but inhibits that of osteocalcin and Cbfa1

Figure 3(A) shows Northern blot analysis performed to determine the effect of EMD on tissue-specific differentiation markers, represented by osteopontin and osteocalcin. Osteopontin was expressed throughout the experimental period, while osteocalcin was expressed only on day 15. As shown in Fig. 3(A), EMD moderately stimulated mRNA expression of osteopontin on days 5 and 10 (131–134% of the control value at 100 μg/ml), but showed no effect on day 15. In contrast, EMD markedly suppressed mRNA expression of osteocalcin at 50–100 μg/ml on day 15. Next, the expressions of Cbfa1 and TGF-β1 mRNA were investigated by RT-PCR (Fig. 3B). Enamel matrix derivative suppressed the expression of Cbfa1 in a concentration-dependent manner throughout the experimental period. However, EMD did not change the mRNA expression of TGF-β1.

### A high level of the active form of TGF-β1 was detected in the conditioned medium treated with EMD

Before starting the experiments, we confirmed the presence of the active form of TGF-β1 in EMD. From the serum-free medium containing 100 μg/ml EMD, 183 pg/ml of active form TGF-β1 was quantified. Although EMD showed no effect on the expression of TGF-β1 mRNA in Fig. 3(B), we detected a fourfold greater amount of the active form of TGF-β1 (67–75 pg/ml) in the conditioned medium treated with 100 μg/ml EMD compared with the control medium (Fig. 4A). In contrast, a large amount of total TGF-β1 was determined at nanogram levels in each culture, and there was no difference in the total

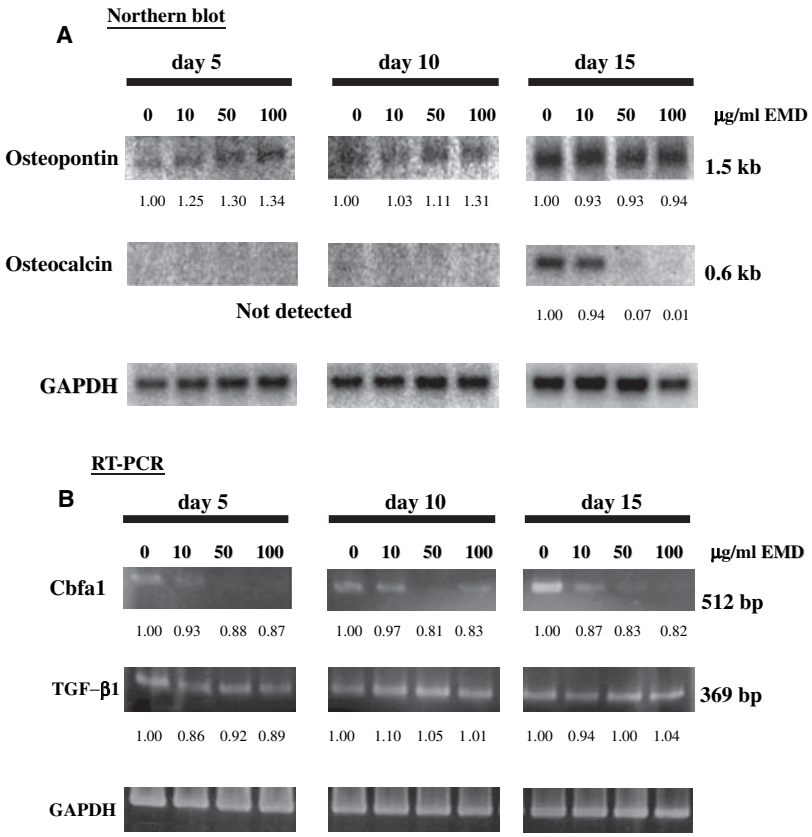


*Fig. 3.* Effect of EMD on mRNA expressions of osteopontin, osteocalcin, Cbfa1 and TGF-β1 in RC cells. The RNA was extracted from RC cells cultured with EMD (10, 50 or 100 μg/ml) for 15 days and analysed by Northern blotting (A, osteopontin and osteocalcin) and RT-PCR (B, Cbfa1 and TGF-β1; 30 cycles). Each mRNA level relative to that of GAPDH mRNA of the control (untreated) cells is shown under the bands.

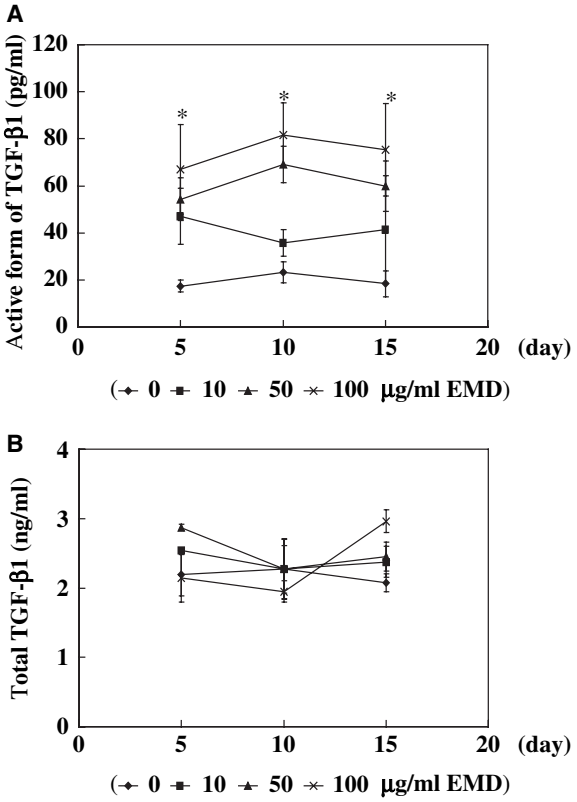


*Fig. 4.* Quantitative analysis of the active form of TGF-β1 (A) and total (latent and active form) TGF-β1 (B) in the conditioned media. The RC cells were cultured with EMD (10, 50 or 100 μg/ml) for 15 days and the concentration of TGF-β1 in the conditioned media was determined using anti-TGF-β1 antibody by ELISA. Values are means ± SE of triplicate samples in three separate experiments. $*P < 0.05$, $**P < 0.01$, EMD vs. control.

amount of TGF-β1 among the four groups (Fig. 4B).

### Neutralization with anti-TGF-β1 antibody partly relieved the ALP activity suppressed by EMD

To determine the action of the active form of TGF-β1 on the differentiation of osteoblastic cells, the effect of anti-TGF-β1 antibody on the EMD-induced inhibition of ALP activity in RC cells was investigated. As shown in Fig. 5, anti-TGF-β1 antibody (0.2 μg/ml) led to a partial recovery of the ALP activity that was suppressed by EMD.

## Discussion

The precise mechanism of periodontal tissue regeneration by EMD has not been fully elucidated. It has been reported that EMD stimulates the proliferation of periodontal ligament cells and affects the differentiation of cementoblasts (11–18). In contrast, EMD inhibits the proliferation of gingival fibroblasts (19,23,24). These results are consistent with the findings of the histological analysis of periodontal tissue regeneration by EMD (3,4). Enamel matrix derivative has been shown to repair the infrabony defects of alveolar bone (3,6,7) and experimental bone defects in an animal model (25,26), whereas the *in vitro* findings of EMD effects on osteoblastic cells were variable according to the cell species and/or culture conditions (16,17). The present findings demonstrated that EMD clearly inhibited osteoblastic differentiation in RC cell culture.

The RC cell culture system is a useful model for investigation of osteoblastic differentiation because it is set up as a colony assay for quantitative assessment of the numbers of osteoprogenitor cells within mixed-cell populations (18,27–29). In the long-term culture of RC cells, the stages of differentiation from osteoprogenitor cells through preosteoblasts and early osteoblasts to mature osteoblasts can be estimated by determining the osteoblastic markers expressed in the cells (28). Therefore, this *in vitro* cell culture system may be a suitable model for studying the effects of EMD on osteoblast differentiation.

The concentration of EMD (0–100 μg/ml) administered to RC cells was selected according to previous studies (16,17,19,23,24,30). Enamel matrix derivative inhibited the ALP activity in a concentration-dependent manner throughout the culture period and this inhibition took place from an early stage of culture (Fig. 1). Schwartz *et al.* (17) reported that EMD did not affect ALP activity in the preosteoblastic cell line 2T9, but stimulated it in normal human osteoblast MG63 cells, and that EMD inhibited it at an early stage but stimulated it at a late stage in primary human osteoblast NHOst cells, which mostly contradicts our present findings. In the present study, it seems that EMD affected the population of osteoprogenitors in the early stage of culture to inhibit their differentiation. As a resultant, BN formation was inhibited by EMD as well as ALP activity (Fig. 2). These results suggest that EMD suppressed the differentiation of RC cells at an early stage and that this effect was reflected in the decrease of BN formation.

Although many reports demonstrate the stimulatory effect of EMD on the proliferation of osteoblasts, its effect on osteoblastic cell differentiation is not clear. We speculate that the *in vivo* bone formation induced by EMD may be derived from its stimulatory effect on osteoblast proliferation. When EMD is locally applied to a periodontal defect, it is possible that differentiation of other cells, such as periodontal ligament cells and cementoblasts, may be predominantly induced at the early stage. If bone differentiation proceeds, new formation of periodontal ligament and cementum may be hindered and ankylosis may be induced. In order to form tooth-associated tissues at first, EMD may inhibit

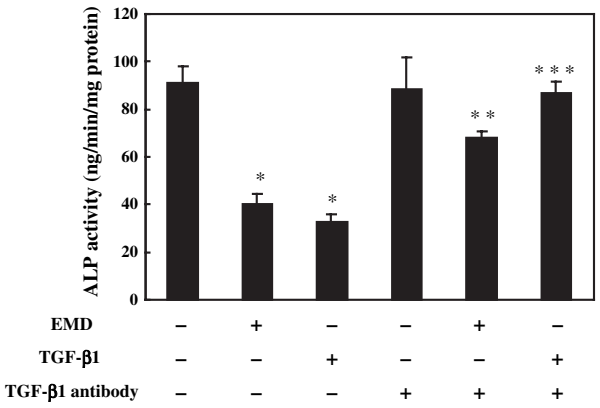


*Fig. 5.* Effect of anti-TGF-β1 antibody on the EMD-induced inhibition of ALP activity in RC cells. The RC cells were cultured with or without EMD (50 μg/ml), TGF-β1 (1 ng/ml) and anti-TGF-β1 antibody for 5 days. The activity of ALP was measured as described in Materials and methods. Values are means ± SE of triplicate samples in three separate experiments. $*P < 0.05$ vs. control, $**P < 0.05$ vs. EMD-treated cells and $***P < 0.05$ vs. TGF-β1-treated cells.

osteoblastic differentiation from the early stage. In the case of socket healing after tooth extraction, it is known that periodontal ligament fibroblasts firstly proliferate and migrate into the coagulum and then osteoblast differentiation takes place (31). Karring *et al.* (32) demonstrated that the repopulation of the root surface by cells from the periodontal ligament might have excluded the possibility for cells derived from the bone to reach contact with the root and thereby to produce ankylosis. From these findings, it is possible that EMD may have a selective function to induce periodontal tissue regeneration by initially inhibiting osteoblastic differentiation.

Although EMD decreased the mRNA expression of osteocalcin on day 15, EMD stimulated that of osteopontin in a concentration-dependent manner on day 5 (Fig. 3A). It has been reported that osteopontin is expressed at an early stage and osteocalcin at a late stage in osteoblastic differentiation (28). Since ALP activity was inhibited by EMD from the start of cultures (Fig. 1), it is thought that subsequent inhibition of osteocalcin expression appeared at the late stage. However, since osteopontin is a multifunctional protein (33), further investigation is necessary to detect the significance of the enhancement of osteopontin mRNA by EMD. Tokiyasu *et al.* (16) reported a similar pattern of expression of osteopontin and osteocalcin in the murine preosteoblast cell line MC3T3-E3 at 100 μg/ml of EMD. Moreover, we found that Cbfa1 mRNA expression was inhibited by EMD in a concentration-dependent manner (Fig. 3B). Core binding factor is an essential transcription factor for osteoblast differentiation and plays a crucial role in osteogenesis (34). This result indicates that EMD inhibited the osteoblastic differentiation of RC cells via the downregulation of Cbfa1.

Recently, it has been demonstrated that EMD contains TGF-β1-like molecules, and this factor may indicate the involvement of EMD in the proliferation of human periodontal ligament cells, oral epithelial cells and gingival fibroblastic cells (19,24). Okubo *et al.* (35) reported that EMD enhanced the expression of TGF-β1 at protein and mRNA levels and that endogenous TGF-β1 was partially related to the EMD-stimulated cell growth. Our results showed that EMD did not alter the TGF-β1 mRNA level throughout the culture period (Fig. 3B), while the concentration of the active form of TGF-β1 was significantly raised in EMD-treated samples (Fig. 4A). We confirmed the presence of the active form TGF-β1 in EMD itself and also in untreated cultures, showing a fourfold greater amount of TGF-β1 in EMD-treated cultures (Fig. 4A), which indicates that three-quarters of the TGF-β1 was derived from EMD and one-quarter from serum contents and/or endogenous TGF-β1 in RC cells. Therefore, the active form of TGF-β1 may be responsible for mediating the effect of EMD and its major origin is EMD itself. Furthermore, we evaluated the association of TGF-β1 with the inhibitory effect of EMD on the differentiation of RC cells and found that anti-TGF-β1 antibody partly reversed the downregulation of ALP activity (Fig. 5). This result indicates that the active form of TGF-β1 in EMD may be partly responsible for the inhibition of differentiation of the RC cells. A previous study showed that continuous exposure to TGF-β1 inhibited RC cell bone nodule formation in a concentration-dependent manner (36) and that TGF-β1 inhibited osteogenic differentiation *in vitro* (37,38).

Taken together, our results show that EMD inhibited the differentiation of RC cells and this effect was partly induced by TGF-β1 as a component of EMD. These inhibitory effects suggest that EMD may have a function initially to inhibit osteoblastic differentiation to permit predominant formation of other periodontal tissues.

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