BASIC RESEARCH-BIOLOGY

The Effect of Transforming Growth Factor Beta 1 on the Mineralization of Human Cementoblasts

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ABSTRACT

Introduction: Transforming growth factor beta 1 (TGF-β1) plays an important role in bone mineralization and has been reported to promote osteoblast proliferation and differentiation. However, there is no report about the effects of TGF-β1 on human cementoblasts. The purpose of this study was to clarify the effect of TGF-β1 on the proliferation and differentiation of the human cementoblast cell line (HCEM) *in vitro*. **Methods:** HCEM cells were stimulated with TGF-β1 at concentrations of 0.05, 0.5, 5, and 10 ng/mL. A proliferation assay was performed from 24–72 hours. The effect of TGF-β1 on mineralization was analyzed by quantifying the area stained with alizarin red on days 7 and 14. Real-time polymerase chain reaction was used to assess the effect of TGF-β1 on the mineralization-related genes alkaline phosphatase, bone sialoprotein, and type I collagen on days 3, 7, and 14. **Results:** TGF-β1 did not affect cell proliferation. TGF-β1 together with the mineralization medium (consisting of ascorbic acid, dexamethasone, and β-glycerophosphate) increased the alizarin red–stained area on days 7 and 14. Real-time polymerase chain reaction revealed that alkaline phosphatase messenger RNA expression was increased in TGF-β1–stimulated HCEM cells in mineralization medium on days 3 and 7, whereas bone sialoprotein and type I collagen messenger RNA expression was increased on day 7. **Conclusions:** Although TGF-β1 does not affect cell proliferation, it does promote cell differentiation and mineralization of HCEM cells. *(J Endod 2021;■:1–6.)*

KEY WORDS

Alkaline phosphatase; bone sialoprotein; cementoblast; transforming growth factor beta 1; type I collagen

SIGNIFICANCE

TGF-β1 does not affect cell proliferation; it does promote cell differentiation and mineralization of human cementoblasts. Mineralization formation after root canal filling is important to activate TGF-β1 in dentin to induce cementoblast differentiation in endodontic therapy.

Pulpitis and apical periodontitis are caused by an endogenous infection of oral flora and cause destruction of pulp and apical periodontal tissue[1]. Removal of the infectious source is essential therapy for these diseases because it promotes periodontal tissue regeneration, which leads to healing[2]. Sealing of the apical foramen via mineralization is considered the optimal healing mechanism for apical periodontal tissue in pulpitis and apical periodontitis. This process involves fibroblasts and osteoblasts of the periodontal ligament, cementoblasts, and undifferentiated mesenchymal stem cells[3].

Root canal preparation during endodontic therapy causes accidents such as root canal wall perforation, ledge, transportation, and root fracture due to the complicated root canal morphology and treatment procedure[4,5]. Recently, mineral trioxide aggregate has been clinically applied as a filling material for the apical retrograde root canal and for sealing the perforated wall of the root canal and has demonstrated an improved healing process [5]. Even in treating accidents such as perforation and root fracture, mineral trioxide aggregate adaptation has enabled conservative treatment by successfully migrating cementoblasts to the root surface and by inducing differentiation and mineralization[6]. Additionally, a bioactive glass–containing root canal filling sealer[7] with excellent biocompatibility induces apatite-like crystal hard tissue in the apex, and migration of cementoblasts on the apical root surface is expected. Furthermore, in a rat apical lesion model in which the apical foramen was destroyed, the addition of a thick new cementum to the apical area was reported using Emdogain (Emdogain Gel; Biora AB, Malmö, Sweden) containing transforming growth factor beta 1 (TGF-β1)[8]. Taken together, the

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TABLE 1 - The Primer Sequences Used in Real-time Reverse Transcription Polymerase Chain Reaction

Gene	Primer forward	Primer reverse	Product length (bp)	Gen Bank accession numbers
ALP	5′-tgcaccatgatttcaccatt-3′	5′-cgttggtgttgagcttctga-3′	154	XM_001826
BSP	5′-gaaccacttccccacctttt-3′	5′-tctgaccatcatagccatcg-3′	201	NM_004967
COLI	5′-ctgaccttcctgcgcctgatgtcc-3′	5′-gtctggggcaccaacgtccaaggg-3′	300	XM_012651
GAPDH	5′-tccaccaccctgttgctgta-3′	5′-accacagtccatgccatcac-3′	450	BC001601

successful healing of apical periodontal tissues requires differentiation into apical foramen and root surface, cementoblast migration, cell proliferation, and cementum and bone formation.

TGF-β plays important roles in tissue development, differentiation, maintenance, and regeneration as well as immune response regulation. The TGF-β family is encoded by 33 different genes and represents cytokines that are distributed across a wide range of tissues and are involved in cell differentiation, migration, adhesion, and extracellular matrix production[9–11]. TGF-β regulates the differentiation and proliferation of cells of bone and cartilage tissues during ontogeny and bone regeneration depending on various factors, such as the level of cell differentiation, the surrounding environment, other cell types, and different growth factors; numerous different responses have been reported previously[12]. TGF-β1, the subject of this study, is the most commonly found isoform in human tissues and is a cell growth factor that plays a central role during tooth development[13,14].

TGF-β1 is present in dentin, cementum, periodontal ligament, and alveolar bone in periodontal tissues and is involved as a growth factor in periodontal tissue regeneration therapy[15,16]. TGF-β1 is a growth factor that plays an important role in bone formation; however, its effects on osteoblast differentiation differ depending on the cell type and the presence of other growth factors[17]. In an animal periodontal tissue defect model, the application of TGF-β1 increased the amount of new bone[18], which is thought to promote the proliferation and differentiation of undifferentiated stem cells present in periodontal ligament tissue[19]. The cementoblasts used in this study originated from pluripotent undifferentiated stem cells and differentiated into cementum. To date, there is no report on the effect of TGF-β1 on human cementoblasts.

The purpose of this study was to determine the effects of TGF-β1 on the proliferation and differentiation of human cementoblasts *in vitro*. The results of this study will contribute to elucidating cementogenesis, which is required for the healing of apical periodontal tissue in endodontic therapy.

MATERIALS AND METHODS

Cell Culture

The human cementoblast cell line (HCEM), immortalized by transfection with the human telomerase transcriptase gene, was generously provided by Professor Takashi Takata (Hiroshima University, Hiroshima, Japan), and the characteristics of HCEM have been described previously[20]. HCEM cells were cultured in α-minimum essential medium (α-MEM; Gibco, Thermo Fisher Scientific, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; BioWest, Nuaillé, France), 100 U/mL penicillin G, and 100 U/mL streptomycin (Sigma-Aldrich, St Louis, MO) at 37°C in an atmosphere of 5% CO_2.

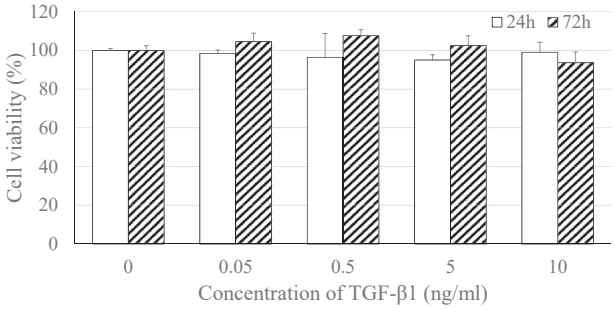

FIGURE 1 – Cell viability of the TGF-β1–stimulated HCEM. HCEM cell viability was assessed after the addition of TGF-β1 (0, 0.05, 0.5, 5, and 10 ng/mL) to the cell culture for 24 and 72 hours. No significant difference was observed in HCEM cells with TGF-β1 stimulation. Furthermore, no cytotoxic effects of TGF-β1 on HCEM cells were observed, even in the presence of 10 ng/mL TGF-β1 at 72 hours.

Proliferation Assay

The proliferation of TGF-β1–stimulated HCEM cells was determined using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega Co, Madison, WI) according to the manufacturer's instructions. In brief, HCEM cells were seeded onto 96-well plates at a concentration of 5×10^4 cells/well in culture medium and incubated for 2 hours to adhere to the plates. The cells were then treated with 0, 0.05, 0.5, 5, or 10 ng/mL TGF-β1 in culture medium and further incubated for 24 or 72 hours. After incubation, cell proliferation was assessed by the assay kit. Results were expressed as mean ± standard deviation of the relative cell viability percentage compared with the negative control ($n = 5$).

Mineralization Assay

To induce mineral nodule formation, HCEM cells were cultured in mineralization medium (MM) composed of α-MEM supplemented with 50 μg/mL ascorbic acid (Sigma-Aldrich), 10^{-8} mol/L dexamethasone (Sigma-Aldrich), and 10 mmol/L β-glycerophosphate (Sigma-Aldrich). HCEM cells were seeded at a density of 5×10^5 cells/well onto 6-well plates and incubated in α-MEM containing 10% FBS until reaching semiconfluence. Cells were then cultured in MM with 5% FBS and stimulated with or without 5 ng/mL TGF-β1. The medium was changed every 3 days, and the cells were maintained for 14 days. On days 7 and 14, mineral nodule formation was detected by staining with alizarin red S (ARS) for calcium. The cells were fixed in 4% formaldehyde neutral buffered solution for 10 minutes and then incubated with 1% ARS in sodium phosphate buffer (pH = 6.2) for 1 hour at room temperature. To quantify mineralization, stained cell areas were analyzed using the image analysis software WinROOF 2015 (Mitani Co, Fukui, Japan).

RNA Extraction and Reverse Transcription Polymerase Chain Reaction Analysis

Total RNA was isolated from HCEM cells cultured in control medium with or without MM

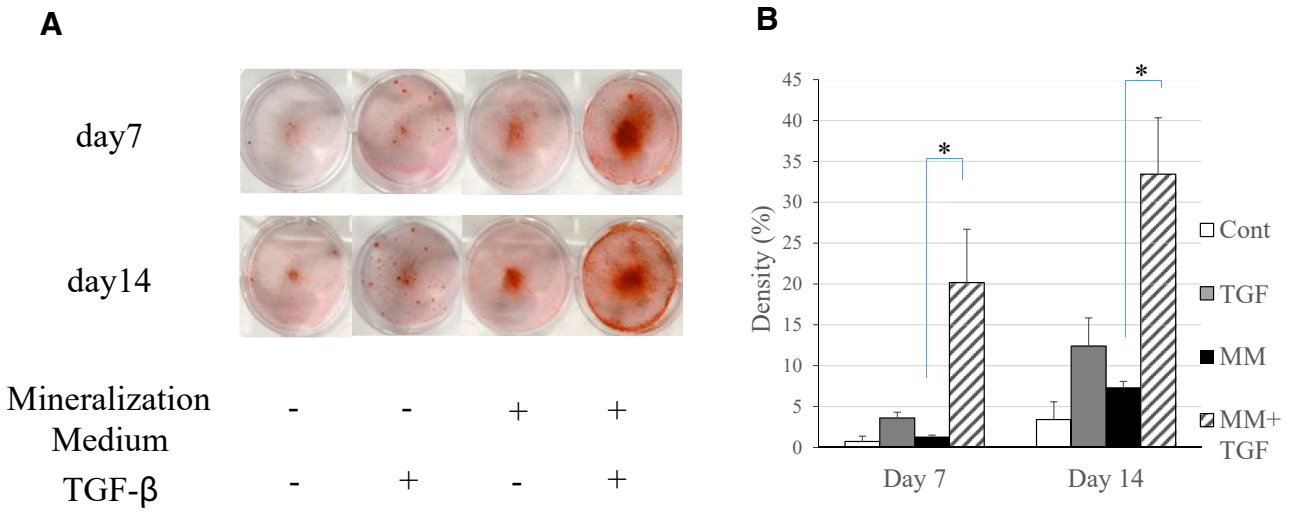

FIGURE 2 – (*A*) The effect of TGF-β1 on HCEM mineralization. TGF-β1 significantly increased the area of ARS-stained red spots in MM, indicating mineralization. (*B*) Quantitative evaluation of the effect of TGF-β1 on HCEM mineralization. *A significant difference between MM and MM with TGF-β1 ($P < .05$).

and TGF-β1 using Isogen (NIPPON Genetics Co, Ltd, Tokyo, Japan) according to the manufacturer's instructions. Complementary DNA was synthesized from 1 μg total RNA using the TaKaRa RNA PCR Kit (AMV) Ver.3.0 (TaKaRa Co, Ltd, Kyoto, Japan). The reverse transcription polymerase chain reaction primers for alkaline phosphatase (ALP), bone sialoprotein (BSP), type I collagen (COL I), and glyceraldehyde-3-phosphate (GAPDH) were purchased from Invitrogen (Tokyo, Japan) and are listed in Table 1. Amplification was performed with an initial denaturation at 94°C for 4 minutes followed by 36 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute, and a final extension at 72°C for 7 minutes. The products were resolved on 2.0% agarose gels and visualized by EZ-Vision DNA Dye as Loading Buffer (AMRESCO, LLC, Solon, OH) under ultraviolet light.

Quantitative Real-time Polymerase Chain Reaction Analysis

HCEM cells were cultured in control medium with or without MM and TGF-β1 for 3, 7, or 14 days, and quantitative real-time polymerase chain reaction for ALP, BSP, and COL I was performed using iTaq Universal SYBR Green One-Step Kit (Bio-Rad Laboratories, Inc, Hercules, CA). The program was set at 95°C for 60 seconds followed by 40 cycles at 95°C for 15 seconds and at 60°C for 40 seconds. Relative gene expression levels were determined using the $2^{-\Delta\Delta Ct}$ method, and the housekeeping gene *GAPDH* was used as an internal control. Quantitative real-time polymerase chain reaction was performed in triplicate.

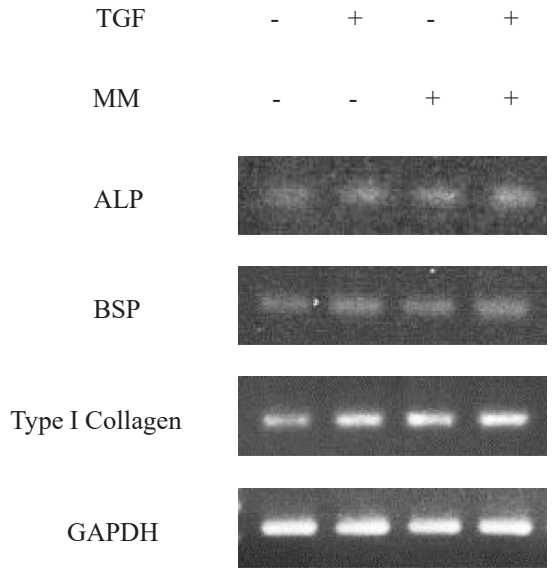

FIGURE 3 – The effect of TGF-β1 (5 ng/mL) on gene expression of mineralization markers and GAPDH in HCEM. HCEM cells were cultured in control medium with or without MM and TGF-β1 for 7 days, and gene expressions of ALP, BSP, and Col I were analyzed by reverse transcription polymerase chian reaction.

Statistical Analysis

The data were analyzed with the Dunnett and Tukey test using SPSS software (Version 22.0; IBM Corp, Armonk, NY). The difference between the experimental groups was considered to be statistically significant at $P < .05$.

RESULTS

TGF-β1 Does Not Affect HCEM Cell Proliferation

HCEM cells were stimulated with different concentrations of TGF-β1 (0, 0.05, 0.5, 5, and 10 ng/mL) for 24 or 72 hours; however, no significant change was observed in the number of cells after TGF-β1 stimulation in either incubation period (Fig. 1).

TGF-β1 Increases Mineralization of HCEM Cells in MM

The ARS method was used to detect calcium ion deposition. On days 7 and 14, nodule formation was confirmed by dark ARS staining in HCEM cells in MM and control medium. On day 7, intense ARS staining was observed in TGF-β1–stimulated HCEM cells in MM compared with that in the control group

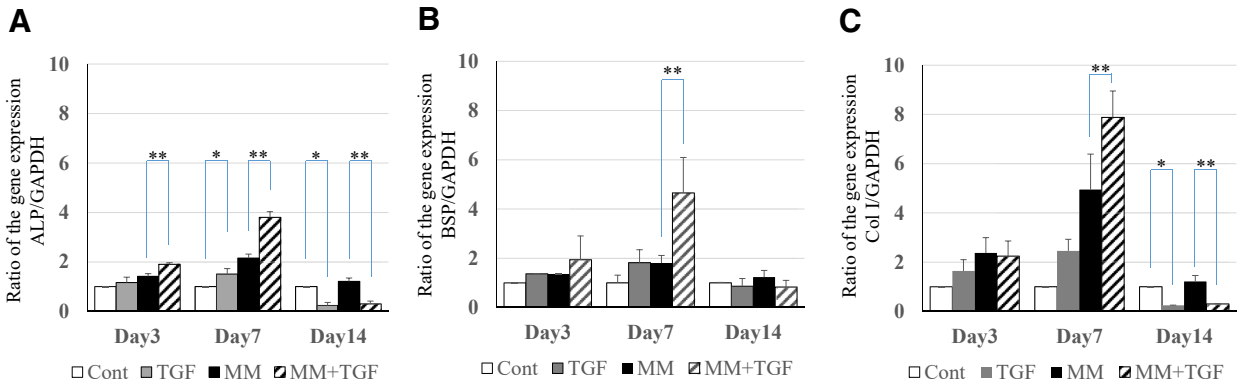

FIGURE 4 – The effects of TGF-β1 on the gene expression levels of mineralization markers in HCEMs. HCEM cells were cultured in control medium with or without MM and TGF-β1 for 3, 7, or 14 days, and the mRNA levels of (*A*) ALP, (*B*) BSP, and (*C*) COL I were analyzed by quantitative real-time polymerase chain reaction. The housekeeping gene *GAPDH* was used as an internal control. *A significant difference between control and control with TGF-β1 ($P < .05$). **A significant difference between MM and MM with TGF-β1 ($P < .05$).

(Fig. 2*A*). On day 14, TGF-β1–stimulated HCEM cells in MM exhibited even stronger staining (Fig. 2*A*). The area of ARS staining was significantly increased in the presence of TGF-β1 and MM on days 7 and 14 (Fig. 2*B*).

Gene Expression

To analyze the effect of TGF-β1 on mineralization-related gene expression in HCEM cells, we first compared the messenger RNA (mRNA) expression levels of ALP, BSP, and COL I by reverse transcription polymerase chain reaction. The cells were incubated in indicated media for 7 days after reaching confluence. HCEM cells expressed mRNA of ALP, BSP, and COL I in control medium without TGF-β1 and MM; however, increased mRNA expressions were observed in HCEM cells with TGF-β1 and MM (Fig. 3). Therefore, we performed quantitative real-time polymerase chain reaction analysis. The cells were incubated for 3, 7, and 14 days.

ALP expression was significantly increased in TGF-β1–stimulated cells in MM but not in the control medium on day 3. ALP expression was significantly increased in TGF-β1–stimulated cells in both MM and control medium on day 7. ALP expression was significantly decreased in TGF-β1–stimulated cells in both MM and control medium on day 14 (Fig. 4*A*). The gene expression level of BSP was significantly increased in TGF-β1–stimulated cells in MM on day 7 but not on days 3 or 14 or in the control medium (Fig. 4*B*). The expression level of COL I increased in TGF-β1–stimulated cells in MM but not in the control medium on day 7. COL I expression was significantly decreased in TGF-β1–stimulated cells in both MM and control medium on day 14 (Fig. 4*C*).

DISCUSSION

In this study, we analyzed the effect of TGF-β1 on HCEM cell proliferation and differentiation. The effects of TGF-β1 on periodontal tissue regeneration have been reported in osteoblasts, periodontal ligament cells, and Hertwig epithelium-derived stem cells at the time of root completion[15–17]. Additionally, investigations on the TGF-β1–stimulated osteoblast cell line MC3T3-E1 demonstrated no effects on cell proliferation[21]. Furthermore, TGF-β1 promotes differentiation but not proliferation of human periodontal ligament cells[22,23]. Our current study demonstrated that various TGF-β1 concentrations exerted no effect on HCEM cell proliferation (Fig. 1). Our results also indicate that TGF-β1 is not cytotoxic to HCEM cells. These findings are consistent with a previous report[21] that demonstrated that TGF-β1 does not affect the cell proliferation of osteoblasts and periodontal ligament fibroblasts in periodontal ligament tissue.

However, our results have shown that TGF-β1 significantly promotes mineralization in HCEM cells. Quantitative real-time polymerase chain reaction revealed that TGF-β1 enhanced ALP mRNA expression on days 3 and 7 as well as BSP and COL I mRNA expression on day 7. Furthermore, TGF-β1 promoted mineralization on day 14 more than on day 7; however, TGF-β1 decreased ALP and COL I mRNA expression on day 14. These results indicate that TGF-β1 does not affect HCEM cell proliferation but does promote HCEM cell differentiation.

It has been reported that during osteoblast differentiation, mesenchymal stem cells differentiate into osteoblast precursor cells via Runx 2 expression and then differentiate into preosteoblasts, osteoblasts, and mature osteoblasts. During early osteoblast differentiation, preosteoblasts express bone protein COL I and ALP mRNA, and during mature osteoblast differentiation and mineralization, ALP mRNA expression decreases, whereas the synthesis of BSP continues[24–28]. HCEM cell differentiation–related gene expressions are comparable to osteoblast differentiation.

TGF-β1 contained in dentin and cementum may work to stimulate the differentiation of cementoblasts and cementum formation. It has been revealed that TGF-β1 contained in dentin is eluted by the EDTA solution used for root canal washing[29]. EDTA treatment just before root canal filling is suggested to be effective in inducing mineralization of human cementoblasts. For dental endodontic therapy, cementum and bone formation after root canal filling is the best healing process. For this, it is clinically important to activate TGF-β1 from dentin to induce cementoblast differentiation for endodontic success.

TGF-β was reported to regulate cementum formation through the Smad transmission pathway in mice[30]. In order to analyze the mechanism of the effect of TGF-β on human cementoblast differentiation, further studies may be required to elucidate the signaling pathway. However, TGF-β1 is histologically distributed in the odontoblast layer adjacent to Hertwig epithelium–derived stem cells during root formation and is present in cementum and dentin[31] Hertwig epithelium–derived stem cells play an important role in root growth. Because TGF-β1 acts on mineralization during tooth

development and is present in cementum and dentin, it plays an important role in periodontal tissue regeneration and the healing and regeneration of apical periodontitis.

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The authors deny any conflicts of interested related to this article.

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