Original

TGF-β abrogates the LPS-induced activation of the NF-κB-mediated signaling pathway that suppresses osteogenic activity in human mesenchymal stem cells

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Abstract：Transforming growth factor-beta（TGF-β）is known to be an important factor for osteogenic differentiation of mesenchymal stem cells（MSCs）. We previously reported that TGF-β1 promoted the human MSC（hMSC）cell line UE7T-13 in an extracellular signal-regulated kinase（ERK）1/2-dependent manner. In addition, ascorbate, dexamethasone（Dex）, and β-glycerophosphate（β-Gp）are widely used for inducing osteogenic differentiation of osteoblast progenitor cells by activating the ERK1/2-mediated signaling pathway. Conversely, lipopolysaccharide（LPS）generally suppresses osteoblastic activity in MSCs. However, the molecular mechanisms underlying the LPS-promoted suppression of osteoblastic differentiation of hMSCs remains to be clarified. This study aimed to 1）identify key molecules that relay intracellular signals for the LPS-induced inhibition of osteogenic activity in hMSCs, and 2）investigate how TGF-β1 affects the LPS-induced inhibition of osteoblastic differentiation of hMSCs. Here, we demonstrated that LPS suppresses ascorbate-, Dex-, and β-Gp-induced osteogenic activity, but not ascorbate-, Dex-, β-Gp-, and TGF-β1-induced osteogenic activity in UE7T-13 cells. In addition, LPS suppressed ascorbate-, Dex-, and β-Gp-induced ERK activation, and partially suppressed the ascorbate-, Dex-, β-Gp-, and TGF-β1-induced ERK1/2 activation in UE7T-13 cells in a nuclear factor kappa-B（NF-κB）-dependent manner. These results suggested that TGF-β1 abrogated the LPS-induced activation of the NF-κB-mediated signaling pathway that relays the suppressive effect on the osteogenic activity of hMSCs partially by the ERK1/2-mediated signal. However, the network would be complexed, and the further research is needed to confirm this.

The present findings partially clarify the molecular mechanisms underlying the development of apical periodontitis-induced suppression of ossification around the tooth root apex, and may aid in

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identifying therapeutic targets for this condition.

Keywords: transforming growth factor-β, lipopolysaccharide, osteoblastic differentiation, mesenchymal stem cell, extracellular signal-regulated kinase

Introduction

Formation of bone nodules in osteoblasts, which is recognized as a marker of extracellular matrix mineralization by osteoblasts, was observed in cultures with ascorbate, dexamethasone, and β-glycerophosphate, an inorganic phosphate source for mineralization in osteoblast cultures (1). In addition, ascorbate, dexamethasone, and β-glycerophosphate are widely used in osteoblast cultures for the induction of osteoblastic differentiation from osteoblast progenitor cells (2). Intriguingly, osteogenic medium (OGM) containing ascorbate, dexamethasone, and β-glycerophosphate induces osteoblastic differentiation of hMSCs in a mitogen-activated protein kinase (MAPK) kinase (MEK) /extracellular signal-regulated kinase (ERK) 1/2-dependent manner (3).

The transforming growth factor-beta (TGF-β) superfamily activates signaling events by binding to respective type I and type II receptors in the extracellular domain. Then, the two type I receptors and two type II receptors construct a tetrameric complex. In this ligand-bound complex of type I and type II receptors, type II receptor kinase activates the type I receptor kinase. The type I receptor kinase activates intracellular signaling by phosphorylating the receptor-regulated Smads (R-Smads), known as canonical TGF-β signaling members (reviewed in references 4). Smads consist of three groups. The first group is composed of the R-Smads; Smad1, Smad5, and Smad8 are activated by the bone morphogenetic protein (BMP) -stimulated type I receptors, whereas Smad2 and Smad3 are activated by TGF-β-stimulated type I receptors. The second group comprises the common mediator Smad (Co-Smad; e.g., Smad4). The third group involves the inhibitory Smads (I-Smads; e.g., Smad6, and Smad7). Activated R-Smads form complexes with the Co-Smad, which enter the nucleus, and together with other cooperative proteins, positively or negatively regulate the transcription of specific target genes. I-Smads suppress the activation of R-Smads by competing with R-Smads for type I receptor interaction and by recruiting specific ubiquitin ligases, resulting in their proteasomal degradation (reviewed in reference 5). TGF-β also stimulates intracellular signal transduction pathways mediated by non-Smad2/3 (non-canonical) TGF-β signaling members, including MEK/ ERK1/2, p38 MAPK, and c-jun N-terminal kinase (JNK), which are activated by TGF-β-activated kinase (TAK) -1 (reviewed in references 6, 7).

Lipopolysaccharide (LPS) is a major component of the outer membrane of gram-negative bacteria (reviewed in reference 8). LPS binds to Toll-like receptor 4 (TLR4), a member of the TLR family (reviewed in reference 9). Dimerization of TLR4 is induced by the recognition of LPS by the TLR4 receptor system, then the LPS-induced intracellular signals mediated by dimerized TLR4 lead to the expression of pro-

inflammatory cytokines and interferons. TLR4 recruits MAL/TIRAP and MyD88 to their TIR domain for activation of nuclear factor kappa B (NF-κB) and MAPKs that regulate pro- and anti-inflammatory cytokine production. TLR4 also recruits TRAM and TRIF, which activate IRF3 and delay activation of MAPKs and NF-κB for the production of type I IFNs (reviewed in references 10, 11).

Interestingly, Huang *et al.* demonstrated that LPS suppresses the BMP-induced osteoblastic differentiation of MSCs by disrupting BMP-promoted Smad1/5/8-mediated signaling (12). In addition, Daigang L *et al.* reported that LPS inhibited the BMP-induced osteoblastic differentiation of preosteoblasts by disrupting BMP-promoted Smad1/5/8-mediated signaling in MEK/ERK1/2- and p38 MAPK-dependent manners (13). We previously demonstrated that OGM supplemented with TGF-β1 clearly promoted osteoblastic differentiation of the human mesenchymal stem cell (MSC) line UE7T-13 in an ERK1/2-dependent manner (14). However, it remains to be clarified how LPS affected the OGM- and TGF-β1-induced osteoblastic differentiation of UE7T-13 cells.

Here, we investigated whether LPS positively or negatively regulated the TGF-β1-induced osteoblastic differentiation of UE7T-13 cells in the OGM by modifying the ERK1/2-mediated signaling pathway. The present study highlights the novel elucidation of the molecular mechanisms underlying the development of apical periodontitis-induced suppression of ossification around the tooth root apex.

Materials and methods

Reagents

Recombinant human TGF-β1 was purchased from PeproTech, Inc. (Rocky Hill, NJ, USA). LPS from *Escherichia coli* (O26: B6) was purchased from Merck KGaA (Darmstadt, Germany). The p38 MAPK inhibitor SB203580, JNK inhibitor SP600125, Smad3 inhibitor SIS3, and TGF-β receptor type I (activin receptor-like kinase 5 (ALK5)) inhibitor SB-431542 were purchased from Calbiochem (Merck KGaA, Darmstadt, Germany). The NF-κB kinase-2 (IKK-2) inhibitor TPCA-1 was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). We confirmed that dimethyl sulfoxide (DMSO), the vehicle used for the SB203580, SP600125, SB-431542, SIS3, and TPCA-1 treatments, did not affect the expression of the osteoblast marker alkaline phosphatase (ALP) or TGF-β1 (data not shown).

Cell culture

The human bone marrow-derived MSC cell line UE7T-13 was purchased from the Health Science Research Resources Bank (JCRB no. 1154, Japan Health Sciences Foundation, Tokyo, Japan). The UE7T-13 cells were cultured in the growth medium [Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS)] at 37 ° C in a humidified incubator with an atmosphere of 5% CO_2. These cells were then sub-cultured at a ratio of 1:4 when they reached sub-confluency. To induce osteogenic differentiation, the UE7T-13 cells were cultured in 6 cm culture dishes, and 12-well culture plates (Nunc, Roskilde, Denmark) with OGM [α-MEM (FUJIFILM Wako Pure Chemical Corp.) supplemented with 50 μg/mL sodium ascorbate (Nacalai Tesque, Kyoto, Japan), 100 nM dexamethasone (Dex; Sigma, St. Louis, MO, USA), 10 mM β-glycerophosphate (β-Gp;

Sigma, St. Louis, MO, USA), and 10% FBS (PAA Laboratories, Piscataway, NJ, USA)]. Some cells cultured in the OGM were further treated with TGF-β1 (1-5 ng/mL) to enhance osteoblastic differentiation induction. In addition, some cells were pretreated with various inhibitors of cellular signal molecules 30 min before the induction of osteogenic differentiation. LPS was also added to the cell culture at the beginning of osteogenic induction, when needed.

RNA isolation and RT-qPCR

UE7T-13 cells were seeded into 12-well tissue culture plates at a density of 1×10^5 cells/well in UE7T-13 growth medium and maintained for 72 h. Subsequently, the cells were cultured in OGM supplemented with or without TGF-β1, and/or LPS at the indicated concentrations for the indicated periods. Some cells were pretreated with various inhibitors of cellular signal molecules 30 min before TGF-β1 administration. Total RNA was isolated from UE7T-13 cells using the ISOGEN reagent (Nippon Gene, Toyama, Japan) according to the manufacturer's protocol. First-strand cDNA was synthesized from total RNA using the PrimeScript RT reagent Kit (Takara-Bio, Shiga, Japan). PCR was subsequently performed on a Thermal Cycler Dice Real Time System (Takara-Bio) using SYBR Premix Ex Taq II (Takara-Bio), with the following specific oligonucleotide primers: human *alkaline phosphatase* (*ALP*), 5-GGACCATTCCCACGTCTTCAC-3 (forward) and 5- CCTTGTAGCCAGGCCCATTG-3 (reverse); human *TGF-β1*, 5-GCGACTCGCCAGAGTGGTTA-3 (forward) and 5-GTTGATGTCCACTTGCAGTGTGTTA-3 (reverse); and human *GAPDH*, 5-GCACCGTCAAGGCTGAGAAC-3 (forward) and 5- ATGGTGGTGAAGACGCCAGT-3 (reverse). The mRNA levels of *ALP* and *TGF-β1* were normalized to *GAPDH* mRNA levels, and the relative amount of each mRNA in each sample was calculated using the $2^{(-\Delta\Delta C(T))}$ method (15). The relative expression levels were expressed as the fold increase or decrease relative to the control.

Western blot analysis

Cells were seeded into a 6 cm culture dish at a density of 3×10^5 cells/well in UE7T-13 growth medium and maintained for 24 h. Afterward, the cells were starved for 24 h as described above and then cultured in OGM supplemented with or without TGF-β1, and/or LPS at the indicated concentrations for the indicated periods. Then, cells were lysed in RIPA buffer [Sigma, St. Louis, MO, USA; 50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS] or lysis buffer [20 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100] containing protease and phosphatase inhibitor cocktails (Sigma). The protein content of the cell extracts was measured using BCA reagent (Pierce, Rockford, IL, USA). Extracts containing equal amounts of protein were separated on 10% SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). After blocking the membranes with 1% BSA or 1% skim milk in T-TBS (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, and 0.05% Tween 20), the membranes were incubated with the appropriate primary antibody. The primary antibodies used were rabbit anti-p44/42 (ERK1/2, #9102), rabbit anti-p38 MAPK (#9212), rabbit anti-SAPK/JNK (#9252), rabbit anti-phospho-p44/42 (ERK1/2, Thr202/Tyl204, #9101), rabbit anti-phospho-p38

MAPK (Thr180/Tyr182, #9211), rabbit anti-phospho-SAPK/JNK (Ther183/185, #9251) (1:1,000; Cell Signaling Technology, Beverly, MA, USA). The blots were then incubated with the appropriate alkaline phosphatase-conjugated secondary antibody, and signals were detected using an alkaline phosphatase substrate kit (BCIP/NBT Substrate Kit; Vector Laboratories Inc., Burlingame, CA, USA).

Statistical analysis

Data are presented as mean ± standard deviation (SD; n = 4) and statistically analyzed by Tukey's multiple comparison test. Values of $^{*}P < 0.01$ and $^{**}P < 0.05$ were considered statistically significant. The results shown in all the experiments are representative of at least two separate experiments.

Results

Ascorbate-, Dex-, and β-Gp-increased expression level of ALP mRNA was further upregulated by TGF-β1 in UE7T-13 cells.

RT-qPCR analysis revealed that sodium ascorbate (50 μg/mL), Dex (100 nM), and β-Gp (10 mM) increased ALP mRNA expression in UE7T-13 cells (Fig. 1A). TGF-β1 (1-5 ng/mL) further upregulated the sodium ascorbate (50 μg/mL)-, Dex (100 nM)-, and β-Gp (10 mM) -increased expression level of ALP mRNA in UE7T-13 cells in a dose-dependent manner (Fig. 1A). In addition, TGF-β1 (5 ng/mL) -upregulated expression of ALP mRNA

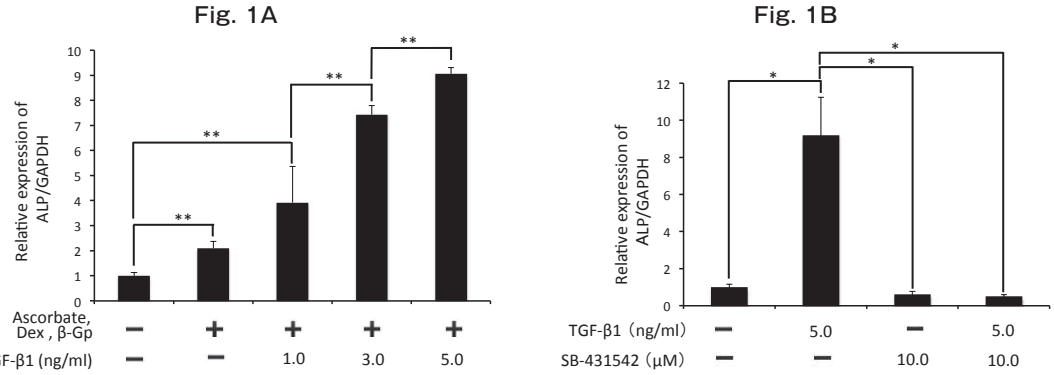

Figure. 1 : *Ascorbate-, Dex-, and β-Gp-increased expression level of ALP mRNA was further upregulated by TGF-β1 in UE7T-13 cells.*
(A) UE7T-13 cells were seeded into 12-well tissue culture plates with growth medium as described in the "Materials and methods" section, and maintained for 72 h. The cells were cultured in osteogenic medium (OGM) containing sodium ascorbate (50 μg/mL), Dex (100 nM), and β-Gp (10 mM) for the induction of osteogenic differentiation of UE7T-13 cells for 72 h. TGF-β1 (1-5 ng/mL) was added to the medium as indicated for further induction of osteoblastic differentiation of the cells treated with the sodium ascorbate, Dex, and β-Gp. (B) UE7T-13 cells were seeded into 12-well tissue culture plates with growth medium as described in the "Materials and methods" section, and maintained for 72 h. The culture medium was replaced with the OGM containing sodium ascorbate (50 μg/mL), Dex (100 nM), and β-Gp (10 mM) for 72 h. For further induction of osteoblastic differentiation of the cells, the cells were treated with TGF-β1 (5 ng/mL). Some cells were pretreated with TGF-β receptor type I (ALK5) inhibitor SB-431542 (10 μM) at 30 min before the TGF-β1 administration. (A, and B) The relative expression level of the ALP mRNA was measured using RT-qPCR analysis. Bars show the mean ± SD (n = 4). $^{*}P < 0.01$, $^{**}P < 0.05$.

in the sodium ascorbate (50 μg/mL) -, Dex (100 nM) -, and β-Gp (10 mM) -treated UE7T-13 cells was clearly abrogated by pretreatment with TGF-β receptor type I inhibitor SB-431542 (10 μM) (Fig. 1B).

LPS suppressed the ascorbate-, Dex-, and β-Gp-induced expression of ALP mRNA in an NF-κB-dependent manner, but not ascorbate-, Dex-, β-Gp-, and TGF-β1-induced expression of ALP mRNA.

We found that LPS (1 μg/mL) significantly suppressed the sodium ascorbate (50 μg/mL)-, Dex (100 nM) -, and β-Gp (10 mM) -induced expression of ALP mRNA in UE7T-13 cells, but LPS (0.1 μg/mL) did not (Fig. 2A). Intriguingly, the IKK-2 inhibitor TPCA-1 (10 μM) significantly abrogated the LPS-induced inhibition of the sodium ascorbate (50 μg/mL) -, Dex (100 nM) -, and β-Gp (10 mM) -induced expression of ALP mRNA (Fig. 2B). However, LPS (0.1-1 μg/mL) did not suppress the sodium ascorbate (50 μg/mL) -, Dex (100 nM) -, β-Gp (10 mM), and TGF-β1 (5 ng/mL) -induced upregulation of ALP mRNA expression (Fig. 2A).

LPS suppressed ascorbate-, Dex-, and β-Gp-induced ERK1/2 phosphorylation, and partially suppressed ascorbate-, Dex-, β-Gp-, and TGF-β1-induced ERK1/2 phosphorylation in an NF-κB-dependent manner in UE7T-13 cells.

Western blot analysis revealed that LPS (10

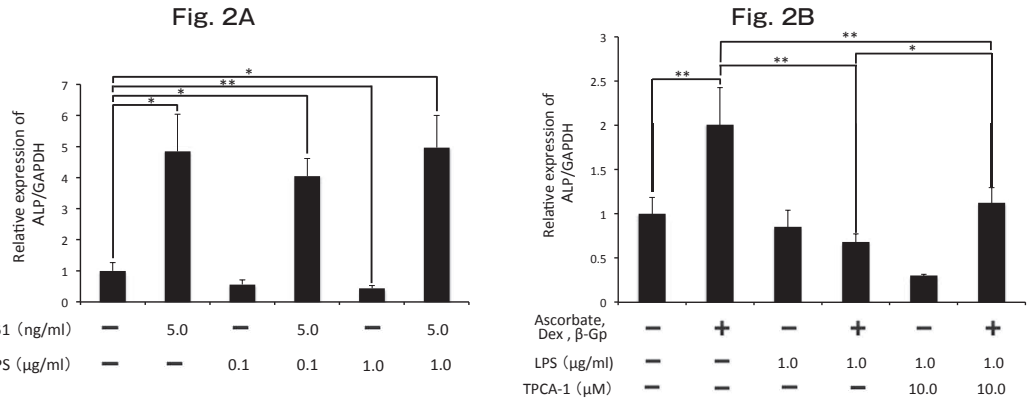

Figure. 2 : *LPS suppressed the ascorbate-, Dex-, and β-Gp-induced expression of ALP mRNA in an NF-κB -dependent manner, but not the ascorbate-, Dex-, β-Gp-, and TGF-β1-induced expression of ALP mRNA.*

(A) UE7T-13 cells were seeded into 12-well tissue culture plates with growth medium as described in the "Materials and methods" section, and maintained for 72 h. The culture medium was replaced with osteogenic medium (OGM) containing sodium ascorbate (50 μg/mL), Dex (100 nM), and β-Gp (10 mM) for 72 h. For further induction of osteoblastic differentiation of the cells, the cells were treated with TGF-β1 (5 ng/mL) as indicated. Some cells were treated with LPS (0.1-1 μg/mL) as indicated. (B) UE7T-13 cells were seeded into 12-well tissue culture plates with growth medium as described in the "Materials and methods" section, and maintained for 72 h. The culture medium was replaced with OGM containing sodium ascorbate (50 μg/mL), Dex (100 nM), and β-Gp (10 mM) for 72 h. Some cells were treated with LPS (1 μg/mL) as indicated. (A and B) The relative expression level of the ALP mRNA was measured using RT-qPCR. Bars show the mean ± SD (n = 4). $^{*}P < 0.01$, $^{**}P < 0.05$.

μg/mL) abrogated the sodium ascorbate (50 μg/mL) -, Dex (100 nM) -, and β-Gp (10 mM) -induced upregulation of ERK phosphorylation level (Fig. 3A). Intriguingly, the LPS-induced suppression of the sodium ascorbate (50 μg/mL) -, Dex (100 nM) -, and β-Gp (10 mM) -induced ERK1/2 phosphorylation was abrogated by TPCA-1 (10 μM) (Fig. 3A). In addition, we found that the phosphorylation level of ERK1/2 began to increase at 90 min, and then peaked at 120 min after stimulation with sodium ascorbate (50 μg/mL), Dex (100 nM), β-Gp (10 mM), and TGF-β1 (5 ng/mL) (Fig. 3B). LPS (10 μg/mL) partially inhibited the sodium ascorbate (50 μg/mL) -, Dex (100 nM) -, β-Gp (10 mM) -, and TGF-β1 (5 ng/mL) -induced upregulation of ERK1/2 phosphorylation at 120 min after the TGF-β1-stimulation (Fig. 3C). Intriguingly, the LPS-induced suppression of sodium ascorbate (50 μg/mL) -, Dex (100 nM) -, β-Gp (10 mM) -, and TGF-β1 (5 ng/mL) -induced ERK1/2 phosphorylation was abrogated by pretreatment with the NF-κB kinase-2 inhibitor TPCA-1 (10 μM) (Fig. 3C).

Both ascorbate-, Dex-, and β-Gp-induced ALP mRNA expression, and ascorbate-, Dex-, β-Gp-, and TGF-β1-induced ALP mRNA expression were mediated by JNK- and p38 MAPK-transduced signaling in UE7T-13 cells.

It remains to be clarified whether the osteogenic activity induced by stimulation with ascorbate, Dex, and β-Gp, and the stimulation

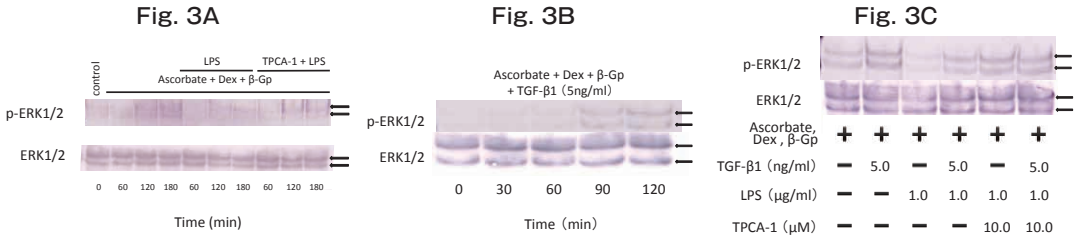

Figure. 3 : *LPS suppressed the ascorbate-, Dex-, and β-Gp-induced ERK1/2 phosphorylation, and partially suppressed the ascorbate-, Dex-, β-Gp-, and TGF-β1-induced ERK1/2 phosphorylation in an NF-κB-dependent manner in UE7T-13 cells.*
(A) UE7T-13 cells were seeded into 6 cm tissue culture dish with growth medium as described in the "Materials and methods" section, and maintained for 72 h. After 48 h starvation, culture medium was replaced with osteogenic medium (OGM) containing sodium ascorbate (50 μg/mL), Dex (100 nM), and β-Gp (10 mM). Then, the cells were maintained for indicated times. Some cells were simultaneously treated with LPS (10 μg/mL) as indicated. In addition, some cells were pretreated with NF-κB kinase-2 inhibitor TPCA-1 (10 μM) at 30 min before the treatment with LPS. (B) UE7T-13 cells were seeded, and maintained, and then starved as described in (A). Then, the cells were treated with sodium ascorbate (50 μg/mL), Dex (100 nM), β-Gp (10 mM), and TGF-β1 (5 ng/mL) for indicated times. (C) UE7T-13 cells were seeded, and maintained, and then starved as described in (A). Then, the culture medium was replaced with OGM containing sodium ascorbate (50 μg/mL), Dex (100 nM), β-Gp (10 mM). Then, the cells were maintained for 120 min. Some cells were simultaneously treated with TGF-β1 (5 ng/mL), or with TGF-β1 (5 ng/mL) and LPS (10 μg/mL) as indicated. In addition, some cells were pretreated with NF-κB kinase-2 inhibitor TPCA-1 (10 μM) at 30 min before the treatment with LPS. (A, B, and C) Expression levels of total ERK1/2 and phosphorylated ERK1/2 were evaluated using western blot analysis as described in the "Materials and methods" section.

with ascorbate, Dex, β-Gp, and TGF-β1 affected the expression status of ALP mRNA in non-canonical pathways such as JNK- and p38 MAPK-dependent manners in UE7T-13 cells. Intriguingly, JNK inhibitor SP600125 (10 μM) abrogated both the sodium ascorbate (50 μg/mL) -, Dex (100 nM) -, and β-Gp (10 mM) -promoted ALP mRNA expression, and the sodium ascorbate (50 μg/mL) -, Dex (100 nM) -, β-Gp (10 mM) -, and TGF-β1 (5 ng/mL) -promoted ALP mRNA expression (Fig. 4A). In addition, p38 MAPK inhibitor SB203580 (30 μM) also abrogated both the sodium ascorbate (50 μg/mL) -, Dex (100 nM) -, and β-Gp (10 mM) -promoted ALP mRNA expression, and the sodium ascorbate (50 μg/mL) -, Dex (100 nM) -, β-Gp (10 mM) -, and TGF-β1 (5 ng/mL) -promoted ALP mRNA expression (Fig. 4B). However, phosphorylation of JNK or p38 MAPK was not detectable by western blot analysis even after stimulation with sodium ascorbate (50 μg/mL), Dex (100 nM), and β-Gp (10 mM), or stimulation with sodium ascorbate (50 μg/mL), Dex (100 nM), β-Gp (10 mM), and TGF-β1 (5 ng/mL) (data not shown). Therefore, it was impossible to determine whether LPS affected the phosphorylation status of JNK or p38 MAPK after stimulation with ascorbate, Dex, and β-Gp, or stimulation with ascorbate, Dex, β-Gp, and TGF-β1. In addition, we found that Smad3 inhibitor SIS3 (10 μM) did not significantly affect the sodium ascorbate-, Dex-, β-Gp-, and TGF-β1-induced

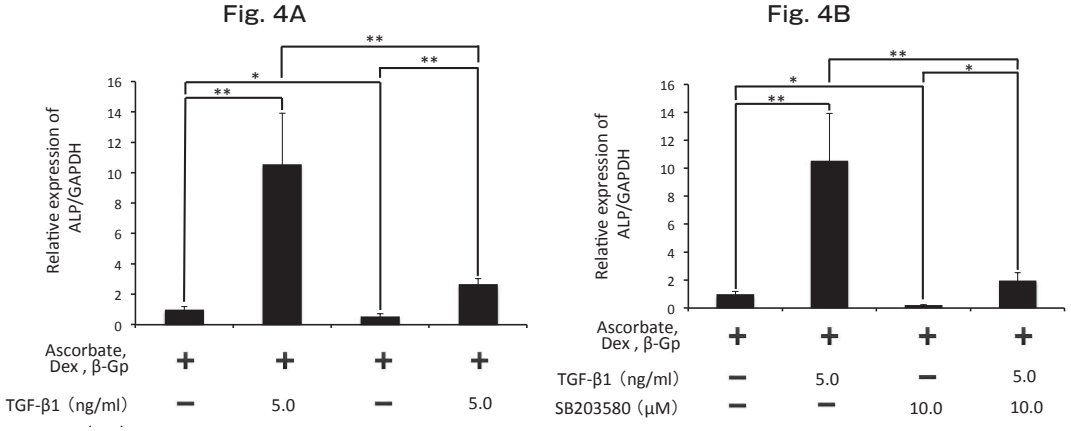

Figure. 4 : *Both the Dex-, and β-Gp-induced ALP mRNA expression, and Dex-, β-Gp-, and TGF-β1-induced ALP mRNA expression were mediated by JNK- and p38 MAPK-transduced signal in UE7T-13 cells.*

(A, and B) UE7T-13 cells were seeded into 12-well tissue culture plates with growth medium as described in the "Materials and methods" section, and maintained for 72 h. The culture medium was replaced with osteogenic medium (OGM) containing sodium ascorbate (50 μg/mL), Dex (100 nM), and β-Gp (10 mM). Then, the cells were maintained for 72 h. Some cells were simultaneously treated with TGF-β1 (5 ng/mL) as indicated. In addition, some cells were pretreated with JNK inhibitor SP600125 (10 μM) (A), or p38 MAPK inhibitor SB203580 (30 μM) (B) at 30 min before stimulation with TGF-β1. The relative expression level of the ALP mRNA was measured using RT-qPCR. Bars show the mean ± SD (n = 4). $^{*}P < 0.01$, $^{**}P < 0.05$.

ALP mRNA expression (data not shown), suggesting that the sodium ascorbate-, Dex-, β-Gp-, and TGF-β1-induced ALP mRNA expression was not mediated by Smad-dependent signaling.

Stimulation with ascorbate, Dex, β-Gp, and TGF-β1 induced the expression of TGF-β1 mRNA.

As shown in Fig. 5, stimulation with sodium ascorbate (50 μg/mL), Dex (100 nM), and β-Gp (10 mM) with or without TGF-β1 (1 to 3 ng/mL) did not upregulate TGF-β1 mRNA expression. In contrast, stimulation with sodium ascorbate (50 μg/mL), Dex (100 nM), β-Gp (10 mM), and TGF-β1 (5 ng/mL) clearly promoted TGF-β1 mRNA expression (Fig. 5).

Discussion

We previously reported that TGF-β1 promoted osteoblastic differentiation of UE7T-13 cells in OGM containing ascorbate, Dex, and β-Gp in an ERK1/2-dependent manner (14), suggesting that ERK1/2-mediated signaling plays an important role in upregulating osteogenic activity in hMSCs. In general, ERK1/2-mediated signaling is essential for the maturation of osteoblast lineage cells; activation (phosphorylation) of ERK1/2 increased the expression of osteoblast marker genes such as *ALP, osteopontin, osteocalcin*, and *Runx2/CBFA1* (16). Yang *et al.* demonstrated that overexpression of the osteogenic transcription factor Runx2/CBFA1 strongly promoted osteoblast marker genes,

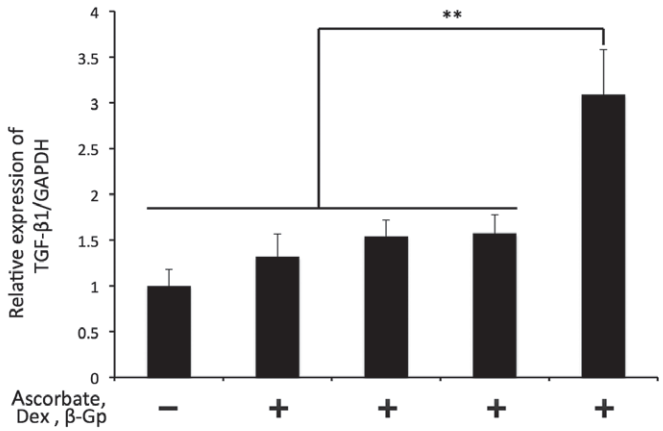

Figure. 5 : *Stimulation with ascorbate, Dex, β-Gp, and TGF-β1 induced expression of TGF-β1 mRNA.*
UE7T-13 cells were seeded into 12-well tissue culture plates with growth medium as described in the "Materials and methods" section, and maintained for 72 h. The culture medium was replaced with osteogenic medium (OGM) containing sodium ascorbate (50 μg/mL), Dex (100 nM), and β-Gp (10 mM). The cells were then maintained for 72 h. Some cells were simultaneously treated with TGF-β1 (1-5 ng/mL) as indicated. The relative expression level of TGF-β1 mRNA was measured using RT-qPCR. Bars show the mean ± SD (n = 4). $^{**}P < 0.05$.

including *ALP*, in mouse MSC cell line C3H10T1/2 (17). Taken together, *Runx2/CBFA1* is likely one of the essential target genes of the ERK1/2-mediated signal for the osteogenic induction of mouse MSCs. Here, we demonstrated that LPS suppressed the ascorbate-, Dex-, and β-Gp-induced osteoblastic differentiation of UE7T-13 cells in an NF-κB-dependent manner (Fig. 2A). In addition, LPS suppressed ascorbate-, Dex-, and β-Gp-induced ERK1/2 phosphorylation in UE7T-13 cells in an NF-κB-dependent manner (Fig. 3A). These results suggested that the LPS-activated NF-κB-mediated signal suppressed ascorbate-, Dex-, and β-Gp-induced osteoblastic differentiation of hMSCs by abrogation of ascorbate-, Dex-, and β-Gp-induced upregulation of ERK1/2 activity. We also found that JNK- and p38 MAPK-activities played important roles in the ascorbate-, Dex-, and β-Gp-induced osteoblastic differentiation of hMSCs (Fig. 4A and 4B). Matsuguchi *et al.* reported that stimulation with ascorbate and β-Gp induced osteoblastic differentiation of mouse primary osteoblasts and mouse osteoblasts MC3T3-E1 cells in a JNK-dependent manner (18). On the other hand, Wang *et al.* reported that stimulation with ascorbate, and β-Gp induced osteoblastic differentiation of mouse primary osteoblasts in a p38 MAPK-dependent manner (19). These results were consistent with our results that the JNK and p38 MAPK pathways played important roles in the osteoblastic differentiation of UE7T-13 cells in OGM containing ascorbate, β-Gp, and Dex. However, it remains to be clarified whether LPS suppressed the ascorbate-, Dex-, and β-Gp-induced osteoblastic differentiation of hMSCs by downregulating of JNK or p38 MAPK activity.

Additionally, TGF-β1 further upregulated ascorbate-, Dex-, and β-Gp-increased expression levels of ALP mRNA in UE7T-13 cells (Fig. 1A). Unexpectedly, LPS did not inhibit the ascorbate-, Dex-, β-Gp-, and TGF-β1-induced upregulation of ALP mRNA expression (Fig. 2A). In contrast, LPS partially downregulated ascorbate-, Dex-, β-Gp-, and TGF-β1-induced phosphorylation levels of ERK in an NF-κB-dependent manner (Fig. 3C). These results suggest that TGF-β1 abrogated the LPS-induced activation of the NF-κB-mediated signaling pathway that relays the suppressive effect on the osteogenic activity of hMSCs partially by the ERK1/2-mediated signal, however, the network would be complexed, and the further research is needed to confirm this. We also found that stimulation with ascorbate-, Dex-, β-Gp-, and TGF-β1 induced ALP mRNA expression in UE7T-13 cells in JNK- and p38 MAPK-dependent manners (Fig. 4A, and 4B). Açil *et al.* reported that combined addition of BMP-2 to OGM containing ascorbate, β-Gp, and Dex produced more potent osteoblast differentiation of hMSCs (20). Fuchigami *et al.* reported that stimulation with ascorbate, Dex, β-Gp, and BMP-9, which belongs to the TGF-β family, potently promoted osteogenic differentiation of human periodontal ligament fibroblasts in ERK1/2-, JNK-, and p38 MAPK-dependent manners (21). These results were consistent with our results that the JNK and p38 MAPK pathways played important roles in the ascorbate-, β-Gp-, Dex-, and TGF-β1-induced osteoblastic differentiation of UE7T-13 cells. However, it remains to be clarified whether LPS positively or negatively affected JNK- or p38 MAPK-activity in UE7T-13 cells stimulated with ascorbate, Dex, β-Gp, and TGF-β1, possibly resulting in the modulation of osteoblastic differentiation. There is a

possibility that TGF-β1 resisted the LPS-induced suppression of osteogenic activity in UE7T-13 cells through abrogation of the LPS-promoted downregulation of JNK- or p38 MAPK-activity.

We found that high dose TGF-β1 (5 ng/mL) induced TGF-β1 mRNA expression in UE7T-13 cells also treated with ascorbate, Dex, and β-Gp (Fig. 5), suggesting that maturely differentiated osteoblasts retained the ability to vigorously express TGF-β1 but undifferentiated MSCs did not. On the other hand, anti-inflammatory macrophages (M2-M Φ s), which are recruited into inflammatory tissue under stimulation with inflammatory chemokines, generally synthesize and secrete anti-inflammatory cytokines such as TGF-β1 (reviewed in 22). There is a possibility that M2-M Φ s, which are recruited into the apical periodontitis-induced inflammatory tissue, abrogates the LPS-induced suppression of osteoblastic differentiation of MSCs by secreting TGF-β1. TGF-β1-differentiated osteoblasts from MSCs then vigorously secrete TGF-β1, resulting in further osteoblastic differentiation of MSCs around the apical periodontitis-induced inflammatory tissue. Intriguingly, Takizawa *et al.* reported that bone marrow-derived MSCs retained the ability to induce polarization of macrophage lineage blood cells into M2-M Φ s in cell-to-cell contact-independent and -dependent manners under hypoxic culture (23). This finding implicates that administration of the large scale prepared M2-M Φ s with *an ex vivo* coculture system between the macrophage lineage blood cells and the MSCs might rescue the development of apical periodontitis-induced suppression of ossification around the tooth root apex.

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Author's contributions

T, A., S, Y., N, C., and S, K. performed the RT-qPCR and western blot analyses. T, A., and M, N. designed this study. T, A., and M, N. were the major contributors in writing the manuscript. All authors have read and approved the final manuscript.

Competing interests

The authors declare that there are no competing interests associated with this manuscript.

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