Original

Dual Nature of TGF- β 1 in Osteoblastic Differentiation of Human Periodontal Ligament Cells

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Abstract: This study investigated the effect of various treatments with TGF- β 1 in osteogenic differentiation of human periodontal ligament (HPDL) cells.

HPDL cells were treated with single 1 ng/ml, 100 ng/ml or multiple (12-h intervals) 1 ng/ml TGF- β 1. Alkaline phosphatase (ALP) activity was detected by ALP activity staining at 4 days. Phospho-Smad3 was detected by western blotting and transcription of osteogenic differentiation markers (*RUNX2*, *ALPL*, *IBSP*: bone sialoprotein, *BGLAP*: osteocalcin) were determined by real-time PCR at various culture periods. Cells were pretreated with or without SB505124, a TGF- β /Smad pathway inhibitor, for 1 h before 1 ng/ml TGF- β 1 treatment at 12-h intervals. ALP activity and phosphorylated Smad3 were detected.

Single treatment with 1 ng/ml TGF- β 1 significantly induced Smad3 phosphorylation, subsequent ALP upregulation and transcription of osteogenic differentiation markers, compared to these inductions with 100 ng/ml TGF- β 1 treatment. In multiple treatments with 1 ng/ml TGF- β 1 at 12-h intervals, only the first treatment significantly induced Smad3 phosphorylation but not the succeeding treatments. Inductive effects of single 1 ng/ml TGF- β 1 in osteogenic differentiation were almost completely abolished by multiple treatments. Furthermore, multiple treatments with 1 ng/ml TGF- β 1 did not induce ALP activity although the TGF- β /Smad pathway was inhibited by SB505124.

Single treatment with low dose TGF- β 1 significantly induced osteogenic differentiation. Multiple treatments inhibited osteogenic differentiation despite inhibition of TGF- β /Smad pathway. These findings suggest that TGF- β 1 both positively and negatively regulates osteogenic differentiation and the inhibitory effect may be mediated by the non-Smad signaling pathway in HPDL cells.

Key words: TGF- β 1, Periodontal ligament, Osteogenesis, Smad3 protein, Phosphorylation

Introduction

The periodontal ligament (PDL) is a connective tissue consisting of heterogeneous population which includes undifferentiated mesenchymal cells, osteoprogenitors, and fibroblasts. Cells in PDL exhibit the potential to differentiate into osteoblasts *in vitro*.¹⁾ A recent study reported that human PDL contains stem cells and consequently PDL stem cells have the potential to generate cementum/PDL-like tissue.^{2,3)} Therefore, PDL is featured as a useful source of regenerative therapy.

Transforming growth factor- β 1 (TGF- β 1) plays a pivotal role in connective tissue regeneration and bone remodeling. Previous

reports demonstrated that TGF- β 1 has significant effects in osteogenic differentiation and bone formation.⁴⁻⁸⁾ TGF- β 1 increased mRNA levels of osteoblastic differentiation markers and alkaline phosphatase (ALP) activity in murine bone marrow stromal cells.⁶⁾ TGF- β 1 was rapidly and transiently increased in orthodontic tooth movement and was associated with bone remodeling *in vivo*.⁹⁾ On the other hand, TGF- β 1 blocked osteogenesis by a variety of mechanisms depending on cell density, TGF- β 1 concentration and differentiation stage of the cells.¹⁰⁻¹²⁾ TGF- β 1 also blocked odontogenesis by downregulation of dentin sialophosphoprotein.¹³⁾ The mitogen-activated protein kinase (MAPK) pathway has been proposed to negatively regulate Smad pathway and osteoblast mineralization.^{14,15)} Some studies reported that TGF- β 1 has biphasic and concentration-dependent effects on

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Table 1. Primer and probe sets for quantitative real-time PCR analysis

Gene symbol	GeneBank accession no.	Primer sequence: sense/antisense	Probe no.	Amplicon
RUNX2	NM_001024630.2	5'-gtgcctaggcgcatctca-3' 5'-gctcttcttactgagagtgaagg-3'	#29	78bp
ALPL	NM_000478.3	5'-caaccctggggaggagac-3' 5'-gcattggtgtgtacgtcttg-3'	#19	78bp
IBSP	NM_004967.3	5'-caatctgtccactcactgc-3' 5'-tcatttgggtgattgcttct-3'	#38	74bp
BGLAP(osteocalcin)	NM_199173.3	5'-tgagagccctcacactctc-3' 5'-accttggctgactctgcac-3'	#81	98bp
SMAD7	NM_005904.2	5'-cgatggattttctcaaccaa-3' 5'-attcgttccccctgttca-3'	#69	74bp
SMAD3	NM_005902.3	5'-ccacgcagaacgtcaaca-3' 5'-gatgggacacctgaacc-3'	#9	72bp
GAPDH	NM_002046.3	5'-agccacatcgctcagacac-3' 5'-gcccaatacagacaaatcc-3'	#60	66bp

osteogenic differentiation.^{10,16,17)} These ambiguous results may be due to variation in the cell models used to analyze TGF- β 1 function.

TGF- β family-induced Smad pathway involve two different classes of serine/threonine kinase receptors called types I and II.¹⁸⁾ TGF- β interact with heterotetrameric complexes of type I and II receptors, which lead to phosphorylation-dependent activation of the dormant type I receptor kinase, in turn, phosphorylated downstream substrates, called receptor-regulated Smads (R-Smads).¹⁹⁾ Smad1, Smad5 and Smad8 are activated by BMPs and Smad2 and Smad3 are activated by TGF- β or activins. These R-Smads, which formed hetero-oligomeric complexes with common Smad (Smad4), translocate to the nucleus where they function as transcription factors via direct DNA binding.²⁰⁾ Inhibitory Smads (I-Smad), including Smad6 and Smad7, act as inhibitors of TGF- β family signaling pathway.^{21,22)} In addition, non-Smad pathways directly or indirectly modify Smad phosphorylation and regulate TGF- β function.²³⁾

Although these reports suggested that TGF- β /Smad pathway could be the major player for induction of osteogenesis, they did not fully explain either the dual effect of TGF- β signaling or the mechanism by which TGF- β 1 influences osteogenesis. To better understand the signaling pathways mediating TGF- β 1 in PDL cells, we examined the activation of Smad pathway and the role of TGF- β 1 in osteoblastic differentiation of human PDL (HPDL) cells.

Materials and Methods

Cell culture and osteogenic differentiation

Normal HPDL cells, defined as pan cytokeratin negative, were purchased from Lonza (Basel, Switzerland) and cultured in BulletKit® Stromal cell growth medium (SCGM, Lonza). Passage was done for 5 to 8 times and cells were seeded at 1×10^5 cells/cm² in SCGM for each assay. Osteogenic differentiation was induced by replacing the osteogenic differentiation medium (OBM), composed of α -MEM (Invitrogen, Carlsbad, CA, USA) supplemented with 5% FBS (Invitrogen), 50 mg/ml L-ascorbic acid (Wako Pure Chemical Industries Ltd., Osaka, Japan) and 10 mM β -glycerophosphate (Wako), with or without rhTGF- β 1 (Wako) which was added the following day. The cells were also treated with or without 10 nM dexamethasone (Dex, Sigma-Aldrich, St. Louis, MO, USA) and 0.5 μ M SB-505124 (Sigma-Aldrich), a competitive inhibitor of activin receptor-like kinase (ALK) 5 that mediates TGF- β -receptor signaling. For single treatment with TGF- β 1, the medium was not changed until day 4. In the process of multiple treatments with TGF- β 1, OBM containing fresh TGF- β 1 was changed every 12 h.

Assay of alkaline phosphatase (ALP) activity

For ALP staining, the cells were fixed in 4% paraformaldehyde for 5 min at room temperature. The cells were then washed with PBS and incubated with ALP substrate solution (Roche Diagnostics, Basel, Switzerland) for 1 h at room temperature. After washing with distilled water for three times, images were captured with phase-contrast microscope. To measure ALP activity, cells

Figure 1. Influence of TGF- β 1 in osteogenic differentiation of HPDL cells. (A) Confluent HPDL cells were cultured in various culture media (SCGM, α -MEM, OBM, OBM + 10 nM Dexamethasone [Dex]) for 4 days and stained for ALP activity. (B) Confluent HPDL cells were cultured in OBM with or without TGF- β 1 (0.1, 1, 10, 50 or 100 ng/ml) for 4 days and were then stained with ALP. Upper panel is the macroscopic appearance of the wells. Lower panel is the phase-contrast microscope images ($\times 100$) of the cells in wells. (C) The cells were cultured in various culture media or various concentration of TGF- β 1 for 4 days after which ALP activity was measured. # $P < 0.001$ compared with SCGM, α -MEM, OBM, OBM+Dex, TGF(10), TGF(50) or TGF(100). & $P < 0.001$ compared with SCGM, α -MEM, TGF(0.1), TGF(1), TGF(10) or TGF(50). (D-F) The mRNA expression levels of osteogenic differentiation genes Runx2 (D), ALP (E), and BSP (F) in HPDL cells treated with 1 ng/ml TGF- β 1 (TGF[1]) or 100 ng/ml TGF- β 1 (TGF [100]) for 4 days, or untreated with TGF- β 1 (cont), were examined by qRT-PCR. The expression levels of each gene were normalized with GAPDH and a value of 1 was assigned to HPDL cells at day 0. Three wells were performed (A, B). Each experiment was performed in triplicate, and the data represent the means $\pm$ SD. The Bonferroni correction for multiple comparisons was applied. * $P < 0.01$, # $P < 0.001$.

were washed twice with PBS and lysed with lysis buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, complete protease inhibitor mixture and 1 % NP-40). ALP activity was assayed using *p*-nitrophenylphosphate as a substrate and the protein content was measured with DC Protein Assay Kit (BioRad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. ALP activity was expressed as nmol *p*-nitrophenol(pNP)/ min/mg of protein.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cultured cells using QIAzol reagent (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. cDNA was synthesized using high-

capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). qRT-PCR analysis was performed using Premix Ex Taq™ reagent (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions. The target genes were *RUNX2*, alkaline phosphatase (*ALPL*), bone sialoprotein (*IBSP*) and osteocalcin (*BGLAP*). *GAPDH* was used as an internal control. All primers and probes of these target genes presented in Table 1 were designed using Probefinder v2.45 (<http://qpcr.probefinder.com/roche3.html>).

Protein extraction and immunoblotting

The cells were lysed with lysis buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, complete protease inhibitor mixture, 1 mM

Figure 2. Effect of TGF-β1 on Smad3 phosphorylation and mRNA expression and of inhibition of ALK kinase on TGF-β1-induced Smad3 phosphorylation and osteogenic differentiation in HPDL cells. (A) Cells were incubated for 12 h in serum-free medium and were treated with 1 or 100 ng/ml TGF-β1 for the indicated times. Western blot analysis using specific antibodies against phospho-Smad3 and Smad3 was then performed. (B) Cells were incubated in OBM with 1 or 100 ng/ml TGF-β1 for the indicated times. Smad3 mRNA expression was analyzed by qRT-PCR. SMAD3 mRNA expression levels were normalized with GAPDH. A value of 1 was assigned to HPDL cells at 0 min. The cells were pretreated with SB505124 (0.1, 0.5, 1, 5 μM), an ALK5 inhibitor, for 1 h in serum free media before treatment with TGF-β1 (1 ng/ml) together with SB505124. (C) Cells extracts were prepared 30 min after treatment and pSmad3 and Smad3 were analyzed by Western blotting. (D) The cells were stained for ALP activity at 96 h after treatment. The number of wells performed three (D). All experiments were repeated three times and representative results were shown. The data represent the means ± SD. *P<0.01 compared with TGF(100) using Student's t-test.

sodium orthovanadate and 1 % NP-40), and the protein content was measured with DC Protein Assay Kit. Equivalent protein concentrations were resolved on NuPAGE 7% Tris-Acetate gels (Invitrogen) by electrophoresis and transferred to a PVDF membrane. The membrane was probed with anti-phosphorylated Smad3 or anti-Smad3 antibodies (1: 1000 for phosphorylated Smad3 and Smad3; Cell Signaling Technology Inc., Danvers, MA, USA), followed by HRP-conjugated goat anti-rabbit IgG. Bound antibodies were visualized using chemiluminescent substrate (ECL plus, GE Healthcare UK Ltd., Buckinghamshire) and exposed to Hyperfilm ECL (GE Healthcare).

Statistical analysis

Each experiment was repeated three times. When ANOVA indicated differences among the groups, multiple comparisons of each experimental group were performed using Bonferroni test.

Results

Characterization of HPDL cells and the effect of TGF-β1 in osteogenesis

OBM or OBM + Dex treatment significantly increased ALP activity in HPDL cells (Fig. 1A and 1C). TGF-β1 was added to HPDL cells at different concentrations (0, 0.1, 1, 10, 50, and 100

ng/ml). Both the number of ALP positive cells and intensity of staining strongly increased in low dose TGF-β1 treatment (Fig. 1B). ALP activity was strongly induced by 0.1 or 1 ng/ml TGF-β1, but weakly induced by 10 ng/ml or higher TGF-β1 compared to low dose TGF-β1 (Fig. 1C). Runx2 mRNA expression was significantly increased by 1 ng/ml TGF-β1, but was strongly down regulated (to a level much lower than the control group) by 100 ng/ml TGF-β1 treatment (Fig. 1D). Similar results were observed when mRNA expression of ALP or BSP was assayed (Fig. 1E and 1F). Thus, the effect of TGF-β1 in the expression of these osteogenic markers was inversely proportional to the concentration of TGF-β1.

Strong phosphorylation of Smad3 by a low dose of TGF-β1 promoted osteogenic differentiation of HPDL cells

Strong phosphorylation of Smad3 was detected following treatment of cells with 1 ng/ml TGF-β1 for 1 h. This phosphorylation was stronger than that observed for 100 ng/ml TGF-β1 (Fig. 2A). Smad3 mRNA expression was altered by TGF-β1 treatment over time (Fig. 2B). The observed variation in Smad3 protein may be due to changes in the expression level of Smad3 mRNA expression. HPDL cells were treated with 1 ng/ml TGF-β1 in the presence of ALK5 inhibitor SB505124 for 30 min (Fig.

Figure 3. Effect of multiple treatments of low dose TGF- β 1 on Smad3 phosphorylation in, and osteogenic differentiation of, HPDL cells. (A) TGF- β 1 treatment protocol. The filled arrow indicates the time at which 1 ng/ml TGF- β 1 was added. The open arrow indicates the time at which 0.5 μ M SB505124 was added. (a) The cells were exposed to a single 1 ng/ml TGF- β 1. Medium without TGF- β 1 was changed at 11 h (*). (b) The cells were exposed to 1 ng/ml TGF- β 1 at 12 h intervals. (c) The cells were exposed to 1 ng/ml TGF- β 1 at 12 h intervals after pretreatment with 0.5 μ M SB505124 for 1 h. (d) The cells were exposed to 1 ng/ml TGF- β 1 alone first and were subsequently exposed 0.5 μ M SB505124. (B) Cells were incubated for 12 h in serum-free medium and were treated with 1 ng/ml TGF- β 1 for the indicated times. Western blot analysis using specific antibodies against phospho-Smad3 and Smad3 was then performed. (C) The cells were stained for ALP at 96 h after (a), (b), (c) or (d) treatment. (D) Western blot analysis of pSmad3 and Smad3 in cell lysates on (a), (b), (c) or (d) treatment at 13 h after the first TGF- β 1 addition. Positive control (PC) was the cell lysate at 1 h after the first 1 ng/ml TGF- β 1 treatment. The number of wells performed three (C). All experiments were repeated three times, and representative results was shown.

Figure 4. Time course analysis of the effect of TGF- β 1 concentration and schedule of administration in the expression of osteogenic differentiation markers. The cells were treated with a single treatment (ST) or multiple treatments (MT, every 12 h) of 1 ng/ml TGF- β 1 (TGF (1)ST or TGF (1)MT) or a ST of 100 ng/ml TGF- β 1 (TGF (100)ST). Total RNA was extracted from the treated cells at indicated time and the mRNA expression of RUNX2 (A), ALP (B), BSP (C), Osteocalcin (D) and SMAD7(E) was examined using qRT-PCR. The expression levels of each gene were normalized with GAPDH with a value of 1 was assigned to HPDL cells at 0 h. Each experiment was performed in triplicate, and the data represent the means $\pm$ SD. The Bonferroni correction for multiple comparisons was applied. * P <0.001 compared with TGF(100) ST or TGF(1) MT.

2C) or 96 h (Fig. 2D) after pretreatment with SB505124 for 1 h. SB505124 dose-dependently decreased TGF- β 1-induced ALP in parallel with a decrease in pSmad3.

Multiple treatments with low dose TGF- β 1 inhibited osteogenic differentiation

We then tested the effect of repeated treatment with 1 ng/ml TGF- β 1 on HPDL-osteogenic differentiation. As expected, strong phosphorylation of Smad3 was detected after the first treatment with 1 ng/ml TGF- β 1 for 1 h (Fig. 3B). Neither the second nor the third treatments induced much phosphorylation of Smad3 (Fig. 3B and data not shown, respectively). The first treatment with 1 ng/ml TGF- β 1 strongly induced ALP but multiple treatments inhibited ALP expression (Fig. 3C). Inhibition of TGF- β / Smad pathway with SB505124 at every 12 h before TGF- β 1 treatment failed to induce ALP expression (Fig. 3C). Phosphorylated Smad3 was not detected in SB505124 treatment (Fig. 3D).

Single treatment of low dose TGF- β 1 induced RUNX2, ALPL, IBSP and BGLAP expression

As shown in Fig. 4, *RUNX2* and *ALPL* expression began to increase after 24 h or 48 h with single 1 ng/ml TGF- β 1 treatment. The expression of these mRNAs showed little increase following a single 100 ng/ml TGF- β 1 treatment or multiple treatments with 1 ng/ml TGF- β 1. A single 1 ng/ml TGF- β 1 treatment induced all osteogenic differentiation markers studied here while multiple or 100 ng/ml TGF- β 1 treatment reduced them.

SMAD7 is transcriptionally induced by TGF- β and negatively regulated TGF- β / Smad signaling pathway.²¹⁾ *SMAD7* expression was transiently and strongly induced under all conditions of TGF- β 1 treatment at 2 h, and decreased thereafter (Fig. 4E). Following single 1 ng/ml TGF- β 1 treatment, *SMAD7* expression continuously decreased and had almost reached the baseline level after 48 h of treatment. However, following 100 ng/ml or multiple TGF- β 1 treatments, *SMAD7* expression was maintained at higher levels relative to those following a single 1 ng/ml TGF- β 1 treatment at 24 h and 48 h. The sustained high expression level of *SMAD7* may be at least one of the reasons for the observed downregulation of pSmad3 by TGF- β 1 treatments because SMAD7 inhibits TGF- β -receptor-induced Smad3 phosphorylation.

Discussion

In this paper we have shown that TGF- β 1 both positively and negatively regulated osteogenic differentiation of HPDL cells and Smad3 phosphorylation depending on the concentration of TGF- β 1 and its time of administration. HPDL cells used in our study were defined as pan cytokeratin negative cells. Thus they could be heterogenous containing population of mesenchymal cells, osteoprogenitors, and fibroblasts.²⁴⁾ Indeed, we found that HPDL cells expressed *RUNX2* at slightly higher levels than normal human

dermal fibroblasts (data not shown), and we could barely detect ALP activity in these cells (Fig. 1A). Osteoblast differentiation markers were significantly induced when cultured in OBM. Thus, HPDL cells are capable of differentiating into osteoblasts.

A single low dose of TGF- β 1 treatment promoted osteogenic differentiation whereas treatment with repetitive low doses, or a single high dose, of TGF- β 1 had much weaker effects in osteogenic differentiation. Zimmermann *et al.* reported that serum levels of TGF- β 1 were significantly higher in bone fracture patients than in normal controls and that lower serum levels of TGF- β 1 were related to delayed healing of fracture.²⁵⁾ Several other reports also described the key role of TGF- β 1 in matrix production, osteoblast precursor cell recruitment during bone repair and acceleration of bone formation.^{4,25-28)} However, in vitro studies showed that TGF- β 1 could positively and negatively regulate osteoblast differentiation depending on the duration of TGF- β 1 treatment or the differentiation stage of the cells.^{7,11,15,29)} Thus, the exact mechanism by which TGF- β 1 modulates osteoblast differentiation is unclear.

It was inferred from the results that TGF- β 1 might initiate osteogenic differentiation with a single low dose TGF- β 1 treatment of HPDL cells, but repetitive or a single excessive high dose of TGF- β 1 treatment might actively switch off and suppress osteogenesis. This hypothesis is supported by the effect of different TGF- β 1 treatments on Smad3 phosphorylation. Thus, treatment with 1 ng/ml TGF- β 1 induced strong Smad3 phosphorylation, whereas 100 ng/ml TGF- β 1 significantly reduced Smad3 phosphorylation (Fig. 2A). Several previous studies indicated that Smad pathway is a key process by which TGF- β 1 modulates osteogenesis. Overexpression of Smad3 in MC3T3-E1 cells enhanced ALP activity and mineralization.³⁰⁾ Borton *et al.* showed that mice with a targeted deletion of Smad3 were osteopenic, had decreased bone mineral density and a decreased rate of bone formation.³¹⁾ Together with our data (Fig. 2C and 2D), these results suggest that TGF- β /Smad pathway (canonical pathway) plays an important role in osteogenic differentiation of HPDL cells. Smad phosphorylation decreased after second treatment with 1 ng/ml TGF- β 1 (Fig. 3B). Multiple treatments of TGF- β 1 abolished the upregulation of ALP and osteogenic differentiation marker genes (Fig. 3C and 4). As shown in Fig. 3A, first, we treated the cells with 1 ng/ml TGF- β 1 alone and then at 12 h later, these cells were treated with TGF- β 1 with/without ALK5 inhibitor SB505124. We found that osteogenic differentiation was completely abolished regardless of SB505124 treatments (Fig. 3C [b] and [c]). Smad3 phosphorylation was completely inhibited by SB505124 treatment (Fig. 3D). These results indicate that although Smad pathway was not active, the inhibition of osteogenic differentiation occurred by multiple treatments. Thus, we suggest that the inhibition of osteogenic differentiation is associated with non-Smad pathway. We hypothesized that there may be a pathway which inhibits

Smad3 phosphorylation that is only activated by a high dose or repetitive TGF- β 1 treatment. This notion is supported by a previous report that Smad3 overexpression greatly enhanced ALP activity that was inhibited by TGF- β 1 treatment.³⁰⁾ Several other studies have reported that TGF- β 1 inhibited osteoblastic differentiation via a non-canonical pathway such as ERK1/2 or JNK pathway.^{14,15,32)} Complex effects of TGF- β 1 on cell function have been widely reported and have been termed “dual effect” of TGF- β 1.^{23,29,33,34)} Non-canonical pathways may play an important role in this dual effect of TGF- β signaling. We hypothesize that TGF- β 1 may have a dual effect in osteogenesis which is either controlling positively or negatively via canonical and non-canonical pathways respectively. Since these non-canonical pathways and their regulation are very complex, further detailed studies are required to fully understand the regulation of osteogenesis by TGF- β signaling.

In conclusion, a low dose of TGF- β 1 induced Smad3 phosphorylation and enhanced the expression of osteogenic differentiation markers. However, repetitive or high dose TGF- β 1 treatment inhibited Smad3 phosphorylation and enhanced the expression of osteogenic differentiation markers. Smad3 phosphorylation plays an important role in osteogenesis whereas the non-canonical pathway may have an inhibitory function. These studies confirmed that TGF- β 1 has a dual effect in osteogenesis and further studies of this dual effect will contribute to a better understanding of the pathological mechanism of periodontitis that will lead to new therapies.

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