Regulation of ALP Activity by TNF-α on Human Dental Pulp

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Abstract

In this study, we examined the effects of TNF-α on Bone morphogenetic protein (BMP-2), Smads (which play intracellular signaling of BMPs) expression and alkaline phosphatase (ALP) activity of human dental pulp (HDP) cells to clarify the mechanism of tertiary dentin formation. The quantity of RT-PCR product for BMP-2 from the HDP cells stimulated by TNF-α is increased. However, ALP activity was not increased on the cells incubated with TNF-α. On the other hand, ALP activity was significantly increased on HDP cells treated with Ammonium Pyrrolidinedithiocarbamate (PDTC, NF- κB inhibitor) groups and combined supplementation of TNF-α and PDTC groups. Furthermore, we examined the effect of TNF-α and PDTC on Smad7 expression using RT-PCR and western blot analysis. Smad7 expression in HDP cells was increased by TNF-α, but decreased by PDTC treatment. These results suggest that NF- κB and Smad7 play an important role in the down regulation of ALP activity by TNF-α on HDP cells. *(J Endod 2006;32:516–520)*

Key Words

ALP activity, BMP, HDP cell, Smad, TNF-α

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The dental pulp is contained in a protective wall of hard tissue. Irritation of the dental pulp by mechanical, thermal, chemical, or bacterial stimuli leads to inflammatory reactions, resulting in the production of proinflammatory cytokines, particularly interleukin-1β and tumor necrosis factor (TNF)-α. An immunohistochemical study has shown that in rats TNF-α-positive cells are present in dental pulp after it has been exposed (1). Inflammation is thought to be closely related to bone metabolism (2, 3). TNF-α, which activates nuclear factor κB (NF- κB), affects bone metabolism, is a potent stimulator of bone resorption, and inhibits osteoblast differentiation. Abbas et al. (4) have reported that TNF-α inhibits the expression of alkaline phosphatase (ALP) and β-glycerophosphate-induced activation of the osteoblast differentiation factor cbfa1. However, its effects on the mineralization of HDP cells have not been fully described. Sakamoto et al. (5) have reported that human dental pulp (HDP) cells show increased ALP activity when exposed to prostaglandin E_2 at low concentrations, whereas the activity is decreased upon exposure to high concentrations. Fukui et al. (6) have reported that TNF-α stimulates the production of bone morphogenetic protein (BMP)-2 by human chondrocytes. BMP-2 has been shown to induce the differentiation of cells of osteoblastic lineage and to enhance their differentiated function. Furthermore, in vivo and in vitro studies have shown that BMP-2 accelerates the differentiation of human dental pulp cells into odontoblasts (7), and in vitro studies have shown that increases their ALP activity (8). In this way, inflammatory cytokines and mediators are thought to play a role in tertiary dental formation, although the mechanism involved has not been fully clarified.

BMPs are growth factors belonging to the TGF-β superfamily, whose members exert their effects via binding to two types of serine/threonine kinase receptors, both of which are essential for signal transduction (9, 10). The type II receptors are constitutively active kinases, which transphosphorylate type I receptors upon ligand binding. The type I receptors activate intracellular substrates such as Smad proteins and determine the specificity of intracellular signals. Eight different Smad proteins have been identified in mammals, and these proteins are classified into three subgroups: receptor-regulated Smads (R-Smads), common partner Smads (Co-Smads), and inhibitory Smads. R-Smads are directly activated by type I receptors, form complexes with Co-Smads, and translocate into the nucleus. Smad1, Smad5, and Smad8 are activated by BMPs, whereas Smad2 and Smad3 are activated by TGF-β and activin. Smad4 functions as a Co-Smad. Structurally, Smad6 and Smad7 are distantly related to the other Smads and have an inhibitory function. One report has indicated that TGF-β/Smad signaling is suppressed by opposing stimuli mediated through the activation of inhibitory Smad7 by NF- κB (Fig. 1) (11).

In the present study, to clarify the effects of TNF-α on the mineralization ability of HDP cells, we measured the expression of BMP-2, Smad1, and Smad7 mRNA and protein in HDP cells stimulated by TNF-α. We also measured the effects of TNF-α and ammonium pyrrolidinedithiocarbamate (PDTC, a NF- κB inhibitor) on ALP activity of HDP cells.

Materials and Methods

Cell Culture

HDP cells were obtained from extracted third molars of young human patients. All molars were extracted during the course of orthodontic treatment, and all patients gave informed consent before providing the samples. After the dental pulp had been extracted under sterile conditions, it was minced, placed on a 35-mm^2

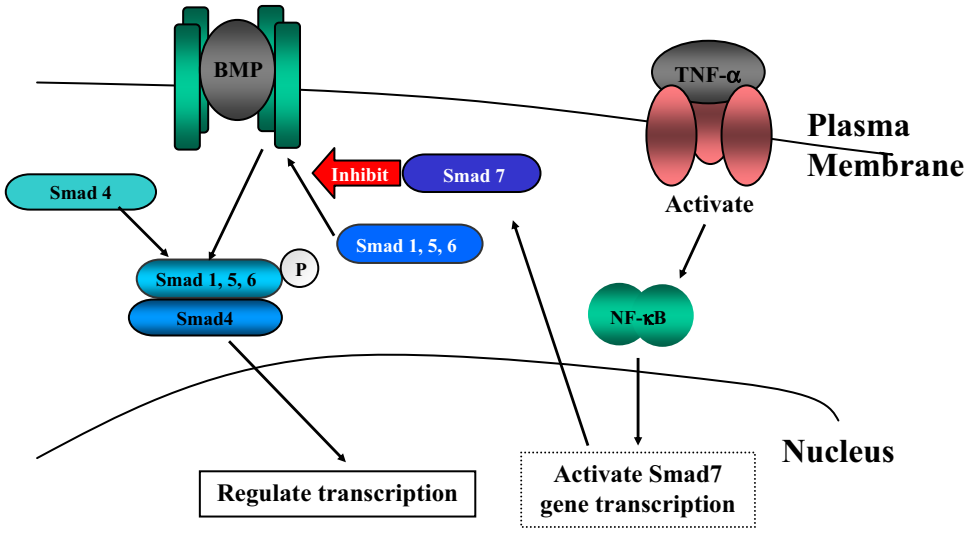

Figure 1. Signaling pathways of BMP, Smad, and TNF-α. BMPs activate Smad1, Smad5, and Smad8, and then Smad1, Smad5, and Smad8 form heteromeric complexes with Smad4, and translocate into the nucleus. The Smad heteromers bind to DNA directly and indirectly via other DNA-binding proteins and thus regulate the transcription of target genes. Smad7 is structurally distantly related to the other Smads and act as inhibitory Smad. TNF-α activates NF-κB, and NF-κB activates Smad7 gene transcription.

tissue-culture dish and then covered with a sterilized glass coverslip. The culture medium used was α-minimum essential medium (Gibco, Grand Island, NY) supplemented with 100 μg/ml penicillin G, 100 μg/ml kanamycin sulfate (Meiji Seika Kaisha, Ltd., Tokyo, Japan), 0.3 μg/ml fungizone (Gibco), 5 mM HEPES buffer (pH 7.2), and 10% fetal calf serum (FCS) under 5% CO_2 in air at 37°C. When cell growth from the explants reached confluence, the cells were detached with 0.05% trypsin (580 BAEE units/mg, Gibco) in phosphate-buffered saline and subcultured in culture flasks. For the experiment, HDP cells from 6 to 9 passages were plated at 5×10^4 cells (0.5 ml medium) per well in 24-well plates.

RNA Extraction

HDP cells stimulated with 10 ng/ml TNF-α or 25 μM PDTC, or a combination of both, were lysed with Trizol (Life Technologies, Inc., Rockville, MD) according to the manufacturer's protocol, and the total amount of RNA was extracted. The final RNA precipitate was stored at −80°C.

RT-PCR

cDNA synthesis and amplification by RT-PCR were conducted using a GeneAmp PCR kit (Applied Biosystems, Inc., Foster City, CA). cDNA synthesis was conducted at 42°C for 15 min in a final 20-μl volume of reaction mixture containing 4 μl of 25 mM $MgCl_2$, 2 μl of 10 × PCR buffer II (500 mM KCl: 100 mM Tris-HCl; pH 8.3) 2 μl of deoxy nucleoside triphosphate (dNTP) (10 mM each), 1 μl of RNase inhibitor (20 units/ml), 1 μl of murine leukemia virus reverse transcriptase (25 units/μl), 1 μl of random hexamers (25 mM), 1 μl of oligo d(T)16 (25 mM), and 2 μl of isolated RNA (1 μg/μl). PCR mixtures containing 4 μl of 25 mM $MgCl_2$, 8 μl of 10 × PCR buffer II, 1 μl of forward primer, 1 μl of reverse primer, 65.5 μl of H_2O, and 0.5 μl of Ampli Taq DNA polymerase were amplified using a GeneAmp PCR system 9600 (Perkin-Elmer Cetus, Norwalk, CT) for 25 cycles consisting of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The PCR primers for amplification of BMP-2 (12), Smad1 (12), Smad7 (13), and glyceraldehyde-phosphate dehydrogenase (GAPDH; as a housekeeping gene) (14) were designed based on the published sequences. The primers were as follows: 5′-GCT GTA CTA GCG ACA CCC AC-3′ (forward primer for BMP-2); 5′-TCA TAA AAC CTG CAA CAG CCA ACT-3′ (reverse primer for BMP-2); 5′-GGC GGC ATA TTG GAA AAG GAG TT-3′ (forward primer for Smad1); 5′-GAG GGG GCC GTG CAG ATG TAT-3′ (reverse primer for Smad1); 5′-GCC CTC TCT GGA TAT CTT CT-3′ (forward primer for Smad7); 5′-GCT GCA TAA ACT CGT GGT CA-3′ (reverse primer for Smad7); 5′-ATC ACC ATC TTC CAG GAG-3′ (forward primer for GAPDH); 5′-ATG GAC TGT GGT CAT GAG-3′ (reverse primer for GAPDH).

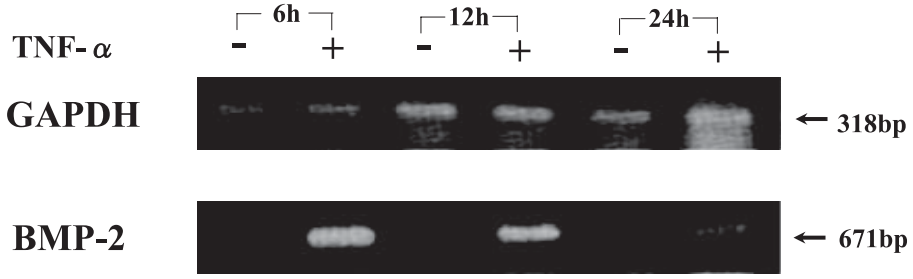

Figure 2. The effect of TNF-α (10 ng/ml) on BMP-2 mRNA expression on HDP cells using RT-PCR analysis. Each PCR product was electrophoresed on agarose gel (1.5%).