

Simvastatin, Cholesterol-lowering Drug, as a New Therapeutic Agent for Periodontal Regeneration

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Abstract : In 1999, Mundy *et al.* has indicated that statins, drugs widely used for lowering serum cholesterol, stimulate bone formation in rodents and increase bone morphogenetic protein (BMP)-2 expression *in vitro*. However, the other growth factors except for BMP-2 and the effects of simvastatin, one of statins, on periodontal tissues have not been shown yet. The purpose of this study was to investigate the possibility of statins as a new effective agent for periodontal regeneration using luciferase reporter gene assay and reverse transcription-polymerase chain reaction (RT-PCR), and the alkaline phosphatase (ALP) activity in human periodontal ligament (HPDL) cells. The results showed that simvastatin induced not only BMP-2 but also transforming growth factor- β 1 (TGF- β 1) in HPDL cells and human osteoblastic cells (NHOst). To examine whether the TGF- β 1 activity was related with productions of the cholesterol biosynthetic pathway, HPDL cells were treated with simvastatin in the presence or absence of mevalonic acid. The TGF- β 1 induction was caused by the inhibition of cholesterol biosynthetic pathway. The differentiation study showed that TGF- β 1 increased the ALP activity, but BMP-2 decreased the ALP activity in HPDL cells, even in the presence of TGF- β 1. The effect of simvastatin is similar to that for BMP-2 with TGF- β 1. The above *in vitro* findings suggest that simvastatin may be effective for periodontal regeneration as new therapeutic agents to induce the regenerative factors such as TGF- β 1 and BMP-2.

Key words: simvastatin, human periodontal ligament (HPDL) cells, transforming growth factor- β 1 (TGF- β), bone morphogenetic protein-2 (BMP-2)

Introduction

Statins, cholesterol-lowering drug, had the ability to stimulate the promoter for BMP-2 in human osteoblasts and promote osteoblast differentiation and mineralization. However, the other growth factors induced by simvastatin except for BMP-2 were unknown and the relative factors of those effects in periodontal ligament cells remain unclear. The purpose of this study is to examine the role of cellular signaling pathway by simvastatin in human periodontal ligament (HPDL).

Materials and methods

Cell Culture

Three periodontally healthy premolars were collected from three patients who had undergone extraction for orthodontic reasons. Informed consent was obtained from all patients under a protocol approved by the Ethics Committee of Tsurumi University. HPDL cells were obtained as previously described by Sommerman *et al.*

Luciferase Reporter Assays for TGF- β and BMP Activities

HPDL cells were seeded at $1.0 \sim 10^4$ cells/well in 96-well plates and incubated for 24 hrs in order to examine TGF- β 1 activity. PAI-1-luc conjugated by TGF- β -responsive PAI-1 promoter region was transfected in HPDL cells by Lipofectamine 2000. HPDL cells were incubated in serum-free medium for 4 hrs during transient transfection and the culture media were replaced with fresh medium containing 2.5% FBS with sample, and then cultured

for an additional 24 hrs. Luciferase reporter assay was performed using the supplier's protocol. We also examined BMP-2 activity using Id1-luc conjugated by BMP-2-responsive Id1 promoter region in HPDL cells.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using RNeasyTM from the HPDL cells which were cultured in the differentiation medium with or without 10^{-6} M of simvastatin. The primer pairs were as follows: TGF- β , 5'-GACATGGAGCTGGTGAAA and 5'-GTATCCGTCTCCTTGGT; BMP-2, 5'-GTGGTGGAAAGTGGCCCACTT and 5'-CTGTTTGTGTTT GGCTTGACG. The PCR conditions were as follows: The PCR started with a 10 minutes denaturation at 94 °C, followed by 30 cycles with denaturation at 94 °C for 30 sec, primer annealing at 55 °C for 30 sec, and product extension at 72 °C for 30 sec; in the final cycle, the 72 °C extension lasted for 7 minutes. The PCR products were analyzed by 4.5% polyacrylamide gel electrophoresis in TBE buffer (pH 8.0). Gels were stained with ethidium bromide, and the bands were visualized under UV light.

Measurement of ALP activity:

HPDL cells, were seeded in 96-well plates, were treated at 80% confluence. The growth medium was then changed to contain which 10nM 1 α ,25-dihydroxyvitamin D₃ in the absence or presence of 10^{-6} M simvastatin. After 10 days of incubation, the cells were washed once with PBS, and ALP activity was determined using 10 mM *p*-nitrophenylphosphate as the substrate in 100 mM 2-amino-2-methyl-1,3-propanediol-hcl buffer (pH 10.0) containing 5 mM MgCl₂ and incubated for 15 minutes at 37°C. Adding NaOH quenched the reaction, and the absorbance at 405

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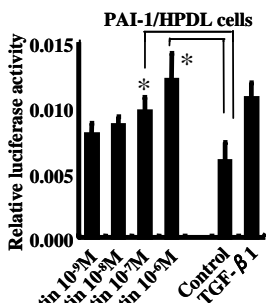


Fig.1 Simvastatin Stimulated Signal Transduction of TGF-β 1

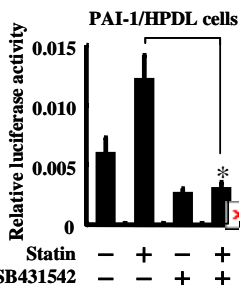


Fig.2 SB431542 Blocked Signal Transduction of TGF-β 1 by Simvastatin

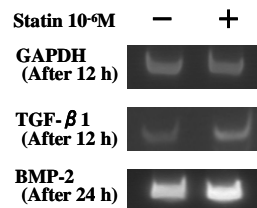


Fig.3 Simvastatin Promotes TGF-β 1 and BMP-2 Expression

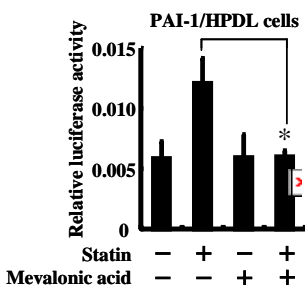


Fig.4 Mevalonic acid inhibits TGF-β 1 induction by Simvastatin

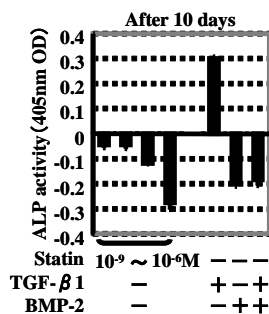


Fig.5 ALP Activities by Simvastatin and Growth Factors

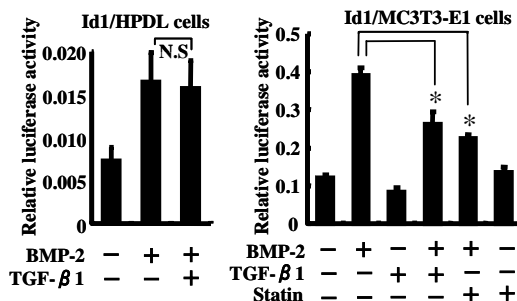


Fig.6 Cross-Talk of TGF-β 1 and BMP-2 in HPDL and MC3T3-E1 Cells

nm was read on a plate reader

Statistical Analysis:

All values are represented as mean ± standard error (SE). Statistical significance was determined by an unpaired Student's t-test, and p < 0.05 was considered statistically significant.

Results

Simvastatin stimulates signal transduction of TGF-β1

Simvastatin stimulated signal transduction of TGF-β1 depend on increasing dose (Fig.1).

SB-431542 blocked signal transduction of TGF-β1 by simvastatin

We treated HPDL cells with 10⁻⁶M of simvastatin in the presence or absence of 10μM of SB-431542. The SB-431542 was identified as an inhibitor of activin receptor-like kinase (ALK) 5. The result showed that simvastatin does not directly stimulate PAI-1 promoter region (Fig.2).

Simvastatin promotes TGF-β1 and BMP-2 expression

RT-PCR analysis detected that simvastatin enhanced the expression of TGF-β1 and BMP-2 (Fig.3).

Mevalonic acid inhibits TGF-β1 induction by Simvastatin

Stimulation of PAI-1 promoter region by 10⁻⁶M of simvastatin was blocked by 1mM of mevalonic acid (Fig.4).

Effects of ALP activities by simvastatin and TGF-β1, and BMP-2

BMP-2 decreased the ALP activity in HPDL cells, even in the presence of TGF-β1. The reaction by simvastatin is similar to the effect for BMP-2 with TGF-β1 (Fig.5).

Cross-talk of TGF-β1 and BMP-2 in HPDL and MC3T3-E1 cells

No reduction of BMP-2 signaling by TGF-β1 was recognized in HPDL cells though BMP-2 signaling was suppressed by TGF-β1 in MC3T3-E1 cells (Fig.6).

Discussion

Simvastatin induced not only BMP-2 but also TGF-β1 in HPDL cells. These TGF-β1 and BMP-2 inductions were caused by the inhibition of cholesterol biosynthetic pathway.

The effects of simvastatin on alkaline phosphatase (ALP) activities were similar to the effect of BMP-2 with TGF-β1, though the reactions for BMP-2 and TGF-β1 on ALP activities were different from HPDL and osteoblast cells (MC3T3-E1 cells) (data not shown). This study, furthermore, showed that signaling for BMP-2 with TGF-β1 was different from HPDL and osteoblast cells. It is considered that the periodontal ligament tissues keep no mineralization under control in spite of situation between bone and cementum tissue because the effect for growth factors in HPDL cells differ from osteoblast cells. In regeneration study, it is important that these effects will be revealed.

The above *in vitro* findings suggest that simvastatin may be effective for periodontal regeneration as new therapeutic agents to induce the regenerative factors such as TGF-β1 and BMP-2. However, because this study is the first step for the clinical application, simvastatin will need more consideration *in vivo*.