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RESEARCH ARTICLE

The Role of NFATc1 on Osteoblastic Differentiation in Human Periodontal Ligament Cells

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치주인대세포의 골모세포 분화에서 NFATc1의 역할

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A recent report showed that nuclear factor of activated T cell (NFATc) 1 is a member of the NFAT family and is strictly implicated osteoblast differentiation and bone formation. Furthermore, the precise expression and function of NFATc1 in periodontal tissue remains unclear. Therefore, the purpose of this study was to investigate the function of NFATc1 in osteoblastic differentiation, and the underlying mechanism regulating periodontal regeneration in human periodontal ligament cells (hPDLCs). NFATc1 messenger RNA (mRNA) and protein levels were accessed by reverse transcription-polymerase chain reaction (RT-PCR) and western blot assay, respectively. Cell proliferation determined using MTT assay. Differentiation was evaluated by alkaline phosphatase activity and formation of calcium nodule with alizarin red S staining. The mRNA expression of osteoblastic differentiation related genes were examined by RT-PCR. Marked upregulation of NFATc1 mRNA and protein was observed in cells grown in osteogenic medium (OS). NFATc1 transactivation was detected in hPDLCs that had been incubated in OS for 14 days. Treatment with 10 μM cyclosporine A (CsA), a known calcineurin inhibitor, reduced the proliferation of hPDLCs, while 5 μM CsA had no effect. Inhibition of the calcineurin/NFATc1 pathway by CsA, attenuated OS-induced osteoblastic differentiation in hPDLCs. In summary, this study demonstrates for the first time that NFATc1 plays a key role in osteoblastic differentiation of hPDLCs and activation of NFATc1 could provide a novel mechanism for periodontal bone regeneration.

Key Words: NFATc1 transcription factor, Osteoblastic differentiation, Periodontal ligament cells

Introduction

Periodontal disease results in the destruction of tooth supporting structures including the cementum, bone, and periodontal ligaments (PDLs). The ultimate goal of periodontal treatment is to regenerate and restore the various periodontal components affected by disease to their original form, function, and consistency[1]. New therapeutic approaches available to achieve periodontal regeneration include use of barrier membranes for guided tissue regeneration, and applying signaling molecules such as growth factors and enamel matrix proteins to root surfaces[2-4]. However, the effectiveness of these approaches is not predictable. Furthermore, the molecular mechanisms by which osteoblastic differentiation is controlled are not completely understood in human periodontal ligament cells (hPDLCs).

The PDL is a fibrous connective tissue that locates between cementum and alveolar bone and is largely composed of cementoblasts, osteoclasts, osteoblasts, and fibroblasts[5]. PDLCs have been shown to exhibits osteoblast-

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like features, such as high alkaline phosphatase (ALP) activity and expression of collagen type I α 1 (ColIα1), osteopontin (OPN), bone sialoprotein, and osteocalcin (OCN)[6]. Additionally, PDLCs can be stimulated to differentiate by a variety of extracellular stimuli, serving to maintain homeostasis or to remodel, repair, and regenerate the surrounding hard tissue[7]. Since a recent study suggested that PDLCs may have important implications for the development of new therapeutic strategies for treating periodontal defects[8]. However, the molecular mechanisms controlling the osteogenic differentiation of PDLC progenitors have not been sufficiently clarified.

The nuclear factor of activated T cell (NFAT) family of transcription factors is consists of five members related to the Rel/NFκB family (NFATc1 to c4 and NFAT5) and is best known that regulate T lymphocyte development and differentiation[9,10]. In unstimulated cells, NFAT is highly phosphorylated and remain in the cytoplasm. When various physiological processes results in an increase in intracellular calcium level, the activation of heterodimeric serine/threonine phosphatase, calcineurin, dephosphorylates NFAT. Then dephosphorylation of NFAT translocate to the nucleus, and induces expression of NFAT target genes[11,12].

NFAT signaling is an important regulator of various biological processes, such as immune development and function[13], cardiac development[14], angiogenesis[15], neural development and function[16] and chondrogenesis[17]. In bone, it is widely accepted that NFATc1 is a master transcriptional factor for induced in osteoclast precursors by receptor activator of NF-κB ligand (RANKL) stimulation[18]. In addition to the regulation of osteoclastogenesis, recent studies indicate that NFAT plays an important role in osteoblast differentiation. Overexpression of NFATc1 in osteoblasts stimulates transcriptional activity of Osterix, which are major osteoblastogenic transcription factors[19]. Patients treated with the calcineurin inhibitors, cyclosporine A (CsA) and FK506, developed osteopenia and showed an increased incidence of fracture[20,21]. Moreover, using low concentrations of CsA have been shown to induce osteoblastic differentiation in vitro and bone mass in vivo[22]. However, the role of NFAT signaling in the osteogenic potential of hPDLCs remains unclear. Thus, the purpose of the present study was to investigate the function of NFATc1 in the osteoblastic differentiation of hPDLCs in vitro.

Materials and Methods

1. Cell culture

Immortalized hPDLCs[23] transfected with human telomerase catalytic component (*hTERT*), were kindly provided by Professor Takashi Takata (Hiroshima University, Hiroshima, Japen). These immortalized hPDLCs displayed spindle-shaped, fibroblastic morphology and strong telomerase activity (data not shown). All cells were cultured in α-modified Eagle medium (α-MEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. Immortalized hPDLCs from passages 70～80 were used in this study. To induce differentiation, cells were cultured with osteogenic medium (OS; 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate) as described previously[24]. α-MEM, FBS, and penicillin/streptomycin were purchased from Gibco BRL Co. (Grand Island, NY, USA).

2. Cell proliferation

Cell proliferation was assessed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich Chemical Co., St. Louis, MO, USA) assay. Briefly, MTT assay solution (1 μg/ml) was added to each 96 well. After a 3-hour incubation period (37°C, 5% CO_2), the supernatant was removed, and the intracellularly stored MTT formazan was solubilized in 200 μl dimethyl sulfoxide. Then optical densities were then measured at 540 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). The percentage of cell viability was calculated as the ratio of the absorbance of treated media that of the control media ×100.

3. Alkaline phosphatase activity assay

For determining ALP activity, the cells were plated in 96-well plates at 1×10^4 cells/well and cultured in OS for 14 days. ALP activity was measured with an ALP fluorometric assay kit (BioVision, Milpitas, CA, USA), following

the manufacturer's protocol. Absorbance was measured at 410 nm by means of an enzyme-linked immunosorbent assay reader (Beckman Coulter, Fullerton, CA, USA).

4. Alizarin red S staining

After 14 days in culture, the cells were rinsed with phosphate buffered saline (PBS), fixed in 70% ice-cold ethanol for 1 h and rinsed with distilled water. Cells were then stained for 10 min with 40 mM alizarin red-S, pH 4.2. The images of alizarin red S staining were photographed with a digital camera.

5. RNA isolation and reverse transcription-polymerase chain reaction

After stimulation, total RNA was extracted from the cells by using Trizol (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions. RNA (1 μg) isolated from each culture was reverse transcribed using oligo $(dT)_{18}$ primers (Roche Diagnostics, Mannheim, Germany) and AccuPower Reverse Transcriptase PreMix (Bioneer, Daejeon, Korea). Thereafter, the RT-generated DNAs (2～5 μl) were amplified with AccuPower PCR PreMix (Bioneer). Primer sequences are detailed in Table 1. PCR products were resolved by electrophoresis on 1.5% agarose gels, and visualized with ethidium bromide.

6. Western blotting

The treated cells were washed with PBS and cytosolic and nuclear protein extracts were prepared using 1× Cell Lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA) supplemented with a protease inhibitor cocktail. Protein concentrations were determined using the Bradford assay (Bio-Rad) as per the manufacturer's protocol. Proteins (30 μg) were mixed with an equal volume of 2× sodium dodecyl sulphate (SDS) sample buffer, boiled for 5 min, and then resolved by SDS-polyacrylamide gel electrophoresis (10% acrylamide) and transferred to polyvinylidene difluoride membrane, immobilon-P (Millipore Co., Milford, MA, USA). Protein bands were detected using an enhanced chemiluminescence system (Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's instructions and exposed to x-ray film.

7. Statistical analysis

The data are expressed as mean±standard deviation of at least 3 independent experiments. Statistical significance was evaluated by one-way analysis of variance with the SPSS ver. 11.0 (SPSS Inc., Chicago, IL, USA) computer program. Statistical significance was determined at $p < 0.05$.

Results

1. Time course change in expression of NFATc1 mRNA and protein during osteoblastic differentiation of hPDLCs

To investigate the expression of NFTAc1 mRNA and protein during osteoblastic differentiation of hPDLCs, hPDLCs were cultured in OS for 14 days and samples

Table 1. Sequences of Oligonucleotide Primer Used for Reverse Transcription-Polymerase Chain Reaction Analysis

Genes	Primer sequence (5'-3')	Annealing temperature (°C)
ColIα1	F: 5'-GGACACAATGGATTGCAAGG-3'	54
	F: 5'-GGACACAATGGATTGCAAGG-3'	
ALP	F: 5'-ACGTGGCTAAGAATGTCATC-3'	55
	R: 5'-CTGGTAGGCGATGTCCTTA-3'	
OPN	F: 5'-CCAAGTAAGTCCAACGAAAG-3'	55
	R: 5'-GGTGATGTCCTCGTCTGTA-3'	
OCN	F: 5'-AGAGCGACACCCTAGAC-3'	57
	R: 5'-CATGAGAGCCCTCACA-3'	
GAPDH	F: 5'-CGGAGTCAACGGATTTGGTCGTAT-3'	62
	R: 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'	

F: forward, R: reverse, ColIα1: collagen Iα1, ALP: alkaline phosphatase, OPN: osteopontin, OCN: osteocalcin, GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

collected were analyzed by reverse transcription-polymerase chain reaction (RT-PCR) (Fig. 1A) and Western blotting. Expression of NFATc1 mRNA and protein was detected that increased in a timedependent manner following exposure to OS until 3 days after the initiation of treatment, after which it decreased. Furthermore, NFATc1 were detected in hPDLCs in both the cytoplasm and in the nucleus. Treatment with OS dramatically increase in nuclear translocation of NFATc1 in hPDLCs (Fig. 1B).

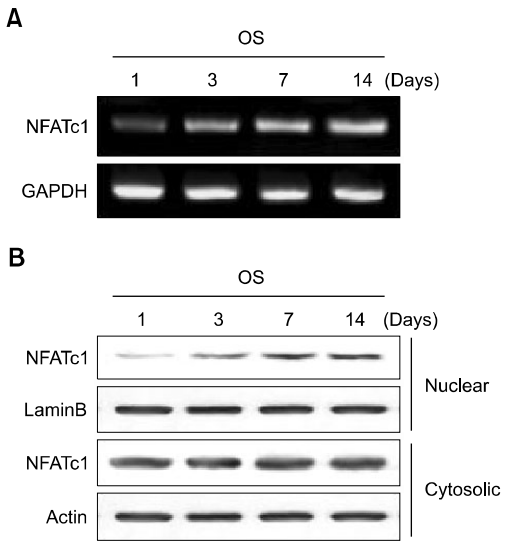

Fig. 1. Time course of NFATc1 messenger RNA and protein expression on osteogenic medium (OS)-induced differentiation of human periodontal ligament cells. (A) Total RNA was isolated and analyzed by reverse transcription-polymerase chain reaction. (B) NFATc1 protein levels were analyzed by western blotting. The data presented are representative of three independent experiments. GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

2. Effects of calcineurin inhibitors, cyclosporine A, on cell proliferation of hPDLCs

We next examined the effect of calcineurine inhibitors, CsA on cell growth in HDPCs. As shown in Fig. 2, OS stimulated cellular viability in a dose-dependent manner after 7 and 14 days of treatment compared with unstimulated

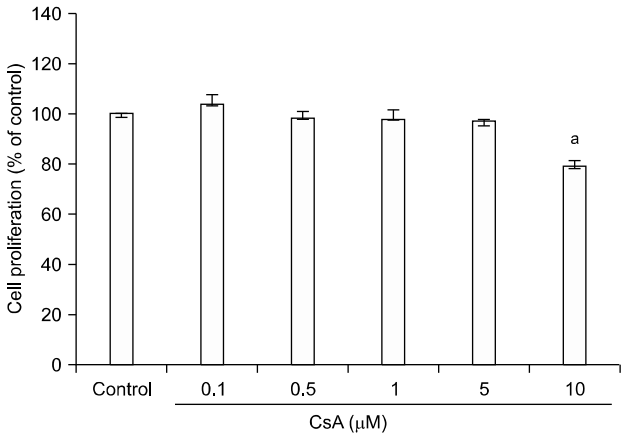

Fig. 2. Effects of calcineurin inhibitors, cyclosporine A (CsA), on cell proliferation of human periodontal ligament cells. Cell proliferation was determined by MTT (n=5) for 14 days. $^{a}p < 0.05$ compared to the control.

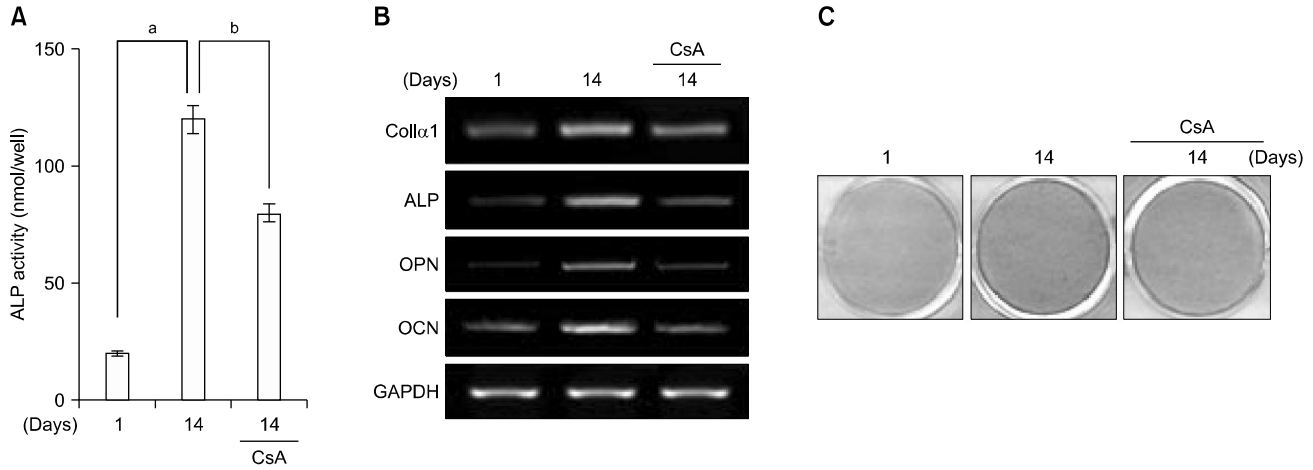

Fig. 3. The effect of calcineurin inhibitor cyclosporin A (CsA) on osteogenic medium (OS)-induced osteoblastic differentiation. Differentiation was assessed by (A) alkaline phosphatase (ALP) activity, (B) reverse transcription-polymerase chain reaction, and (C) alizarin red staining. $^{a}p < 0.05$ compared to the control, $^{b}p < 0.05$ compared with OS-treated. Data are representative of three independent experiments. Collα1: collagen Iα1, OPN: osteopontin, OCN: osteocalcin, GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

control cells. Significant growth stimulatory effects were observed at 10 μM CsA on 14 days. However, in the presence of 5 μM CsA, cell proliferation was not affected by OS treatment.

3. Effect of calcineurin inhibitor, cyclosporine A, on OS-induced osteoblastic differentiation of hPDLCs

ALP is considered to be of osteoblastic differentiation, while OPN and OCN were intermediate and late bone differentiation that OS had stimulated osteoblastic differentiation in hPDLCs. Next, calcium nodule formation by alizarin red staining and the expression of early, intermediate, and late differentiation markers by RT-PCR were examined. As shown in Fig. 3A, ALP activity was significantly decreased by 5 μM CsA. In addition, treatment with the CsA reduced the expression of differentiation marker, ColIα1, ALP, OPN and late OCN mRNA (Fig. 3B). Calcium nodule formation and henced osteoblastic differentiation was confirmed by positive alizarin red staining, and exposure to CsA for 14 days reduced mineral deposition (Fig. 3C).

Discussion

In the present study, we demonstrated the role for the calcineurin/NFAT signaling pathway during the differentiation of hPDLCs, an important subject for successful periodontal tissue regeneration. Additionally, our results indicate that NFATc1 positively regulates expression of osteoblastic differentiation marker in osteogenic-induced condition.

The PDL is a source of pluripotential cells and molecular factors controlling cellular events in the surrounding tissues[8]. The PDL consists of a heterogeneous cell population and human PDL-derived cells have different tendencies for osteogenesis, chondrogenesis, adipogenesis, or proliferation. Osteoblastic differentiation and mineralization typically involve an initial period of cell proliferation and extracellular matrix biosynthesis, followed by cell differentiation[25]. We demonstrated in this study that hPDLCs differentiate into osteoblasts that produce mineralized nodules and express early (ColIα1 and ALP), intermediate (OPN) and late (OCN) markers of osteoblastic differentiation when cultured in osteo-inductive medium for 14 days. The induction of osteoblastic differentiation by this medium was consistent with the results of previous in vitro studies performed using human bone marrow stromal cells[26], MC3T3E1 osteoblasts[27] and hPDLCs[28].

The transcription factor NFATc1, first shown to be important in T cells, is now recognized as a significant regulator of osteoclastic differentiation and has major effects on transcriptional regulation in osteoblasts[19]. To understand whether the NFATc1 involved in osteoblastic differentiation, molecular mechanisms regulating the osteogenic differentiation, we analyzed the change in time course of activation of NFATc1 during 14 days. As the culture progressed, NFATc1 in nuclear were upregulated and widely expressed throughout the differentiation process of hPDLCs. These results are similar to a previous report that baicalein, a naturally occurring compound, stimulates osteoblastic differentiation via activation of NFATc1 in mouse osteoblastic MC3T3-E1 cells[29]. Furthermore, high $[Ca^{2+}]o$ increases the expression level and the transcriptional activity of NFAT in MC3T3-E1 subclone 4 cells.

CsA, widely used immunosuppressive drugs, are known to work by inhibiting the calcineurin/NFATc1 signaling pathway[30]. In addition, our results show that calcineurin inhibition by low concentration of CsA was not influenced osteoblast proliferation in hPDLCs. Interestingly, we also discovered that calcineurin inhibition by CsA significantly reduced OS-induced osteoblastic differentiation in hPDLCs. These findings indicate that activation of NFATc1 signaling is involved in the OS-induced osteoblast differentiation in hPDLCs. Consistently, overexpression of NFAT increased the number of bone nodules and NFATc1 cooperatively enhanced Osterix activation of the ColIα1 promoter, but did not enhance Runx-2 activity, which are major osteoblastogenic transcription factors[19]. In contrast, some studies have reported that CsA and FK506 enhance osteoblastic differentiation and bone formation both in vivo and in vitro[19,31-33]. Mice expressing a dominant negative Nfatc1 in osteoblasts display increased bone volume due to increased bone formation, suggesting that NFATc1 inhibits osteoblastic function[34].

In conclusion, we report that NFATc1 translocation into nuclear promotes osteoblastic differentiation in hPDLCs,

as shown by the induction of ALP activity, formation of mineralized nodules, and upregulation of the expression of marker genes. In contrast, NFATc1 activation by pharmacological inhibitor, CsA, reduced the level of differentiation in cells. The data presented in this study provide that new insight of a novel mechanism for osteoblast differentiation. Our work suggests that the Cn/NFAT signaling pathway plays a critical role in the positive regulation of osteoblast differentiation in hPDLCs.

Summary

Effective regulation of PDLCs contributes to successful periodontal tissue regeneration. Although NFATc1 activation stimulated osteoblastic differentiation in osteoblastic cells, the role of NFATc1 in periodontal regeneration was not completely understood. To our knowledge, this is the first report of the expression of NFATc1 mRNA and protein being induced in hPDLCs during osteoblastic differentiation. NFATc1 inhibition by CsA in hPDLCs decreased cell growth. Furthermore, treatment with CsA blocked the expression of differentiation marker, ALP activity, and mineralization. These findings support the hypothesis that NFATc1 may play an important regulatory role in osteoblastic differentiation for periodontal regeneration.

요 약

치주인대세포의 효과적인 조절은 성공적인 치주 조직 재생에 중요한 역할을 한다. NFATc1의 활성화가 골모세포에서 분화를 자극하지만, 치주인대세포가 골모세포로 분화하는 과정에서 NFATc1의 역할은 아직 보고되지 않았다. 본 연구는 hPDLCs가 골모세포로 분화하는 동안 NFATc1의 mRNA의 발현과 단백질 발현이 유도됨을 처음으로 확인하였다. CsA에 의한 NFATc1의 억제는 세포증식을 감소시켰다. 게다가, CsA를 처리한 결과, 분화표지자, ALP activity 및 광화결정형성을 감소시켰다. 이러한 연구 결과는 NFATc1이 치주 재생을 위한 골모세포 분화에 중요한 조절자 역할을 할 수 있을 것으로 생각된다.

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