치의과학석사학위논문

Effects of calcitonin on dental stem cells derived from human periodontal ligament

칼시토닌이 사람 치주인대유래 치아줄기세포에 미치는 효과

2017 년 8 월

서울대학교 대학원

치의과학과 구강악안면외과학 전공

김 수 호

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Abstract

Effects of calcitonin on dental stem cells derived from human periodontal ligament

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Purpose : Calcitonin is a natural hormone controlling calcium metabolism by decreasing blood calcium ion level and storing calcium deposit in bone. Dental stem cells have been studied in regenerative medicine because of its multi-lineage differentiation potential. The purpose of this study is to determine the effects of calcitonin on human periodontal

ligament stem cells (hPDLSCs). **Materials and Methods** : After primary culture of hPDLSCs from extracted third molar, FACS, Alizarin red S staining, Alcian blue staining, and Oil red O staining were done to confirm stem cell origin and multi-lineage differentiation potential. Effects of calcitonin on proliferation, migration, and differentiation of hPDLSCs were evaluated with CCK-8 assay, wound healing assay, ALP staining, Alizarin red S staining, and real-time PCR analysis. **Results** : Cultured human PDLSCs were confirmed of its mesenchymal stem cell-like properties with osteogenic, chondrogenic, and adipogenic differentiation potential. No significant cytotoxicity was observed after 24 and 48 hours, and cell proliferation increased mostly with 1 nM calcitonin at 48 hours. Under the same concentration, cell migration and expression of osteogenic differentiation markers increased the most. **Conclusion** : From this study, treatment with 1 nM calcitonin promotes osteogenic differentiation, cell proliferation and migration of hPDLSCs. These results support the idea that calcitonin could be used not only in treatment of osteoporosis and hypercalcemia but also in the field of regenerative medicine.

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Keywords : Calcitonin, Dental stem cells, Human periodontal ligament, Osteogenesis, Regenerative medicine

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Introduction

Calcitonin, a natural 32 amino acid peptide hormone produced by parafollicular C-cells in the thyroid gland, is known as one of the hormones considered to influence bone remodelling, and has been preserved during the evolution from marine life to ground dwellers[1)]. After its first discovery in 1962, Potts *et al.* figured out the amino acid sequence of human calcitonin in 1992,; then synthetic salmon calcitonin has been used as a drug

due to similar structure to human calcitonin but with 50-fold higher potency than mammalian calcitonin[2,3].

Calcitonin participates in precise and regulated calcium metabolism, lowering blood calcium ion level and increasing calcium deposit in bone, which stores approximately 99% of calcium in the body, acting as counterpart of parathyroid hormone[4]. It has anti-resorptive effect which is mediated by direct binding to calcitonin receptor on the osteoclast, leading to decrease in blood calcium ion levels[5]. Also, it inhibits reabsorption of calcium ion and phosphate in proximal renal tubular cells, leading to excretion in the urine[6].

Because of this calcium regulation, calcitonin is indicated in the treatment of hypercalcaemia and metabolic bone diseases such as post-menopausal osteoporosis, Paget's disease, and other osteolytic bone tumor and malignancy[7]. Also, various *in vivo* studies demonstrated that calcitonin reduces osteoarthritic cartilage pathology with increased bone turnover by chondroprotective effects[8-10].

Mesenchymal stem cells (MSCs), the multipotent stromal cells, have been researched widely with the rise of regenerative medicine, and dental stem cells are one of the popular sources of mesenchymal stem cell therapies due to their convenient and non-invasive nature[11]. Dental stem cells have been isolated from various parts of teeth subsequently,

starting from pulp tissue, followed by exfoliated deciduous teeth, periodontal ligament, apical papilla, and dental follicle, which are now known for dental stem cell sources[12,13]. Since these dental stem cells are derived from neural crest origin, multi-lineage differentiation potential and proliferative ability of dental stem cells take on different aspects compared with mesenchymal stem cells derived from the bone marrow[13]. Various *in vivo* studies showed the effectiveness of dental stem cell transplantation in regenerating dental tissue and osseous defects caused by oral diseases such as periodontal disease, dental caries, cancers and so on[14,15].

However, the exact mechanism and transcription factors involved in proliferation and differentiation of dental stem cells are still unclear, and diverse molecules have been investigated to determine their influence on proliferation and differentiation efficacy into mesenchymal lineage[16]. Morabito *et al.* demonstrated that calcitonin inhibits proliferation and induces osteogenic differentiation of mesenchymal stem cells from human amniotic fluid (huAFMSCs)[17]. Since little is known about the effects of calcitonin on dental stem cells, this study aimed to elucidate them.

Materials and Methods

Reagents and materials

Salmon calcitonin (sCT) powder was purchased from Sigma-Aldrich (St. Louis, MO, USA). Calcitonin powder was dissolved in the DEPC-treated water (Ambion, Austin, TX, USA), and stored at -20℃. The various concentrations of salmon calcitonin prepared in this study (0 nM, 0.1 nM, 1 nM, 10 nM and 100 nM) were referred from the previous literature[17].

Primary cell cultures

From a 20-year-old male, third molars were collected for cell cultures after obtaining informed consent from the patient. The protocol was approved by the Institutional Review Board of the Seoul National University Dental Hospital, Seoul, South Korea (IRB number 05004).

First, periodontal ligament tissue of the extracted third molar was gently separated with surgical scalpel blade No. 15 and digested in a solution of 3 mg/mL collagenase type I (Worthington Biochem, Freehold, NJ, USA) and 4 mg/mL dispase (Boehringer, Mannheim, Germany) for 1 hour at 37℃. Mixed solutions were passed through a 40 μm strainer (Falcon-BD Labware, Franklin Lakes, NJ, USA) to obtained single-cell suspensions. The obtained cell suspensions were

cultured in the alpha-modification of Eagle's medium (α-MEM; Gibco BRL, Grand island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco BRL), 100 μM ascorbic acid 2-phosphate (Sigma, St. Louis, MO, USA), 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (Biofluids, Rockville, MD, USA) and incubated at 37℃ in 5% CO_2. The medium was changed after the first 24 hours, and every 3-4 days afterwards. The isolated cells formed single cell-derived colonies. All primary cells used in this study were at passage 3 to 4.

Flow cytometry analysis (FACS)

The expression of known human mesenchymal stem cell-associated surface markers was analyzed by flow cytometry analysis (FACS). First, hPDLSCs in their third passage (1.0×10^6 cells) were detached from the culture dish using trypsin / EDTA and washed with PBS. Then detached cells were fixed with 3.7% paraformaldehyde from 95% paraformaldehyde power (Sigma-Aldrich) diluted in phosphate-buffered saline (PBS) (3.7 g/100 mL) for 10 min. To block non-specific antibody-binding sites, the cells were resuspended in PBS containing 1% bovine serum albumin (BSA) (ICN Biomedicals, Irvine, CA, USA) for 30 min, then incubated with specific antibodies against CD34, CD13, CD90, or CD146 at 4℃ for 1

hour. Subsequently, the cells were incubated with fluorescent dye conjugated secondary antibodies at room temperature for another 1 hour in the dark. All antibodies used in FACS were purchased from BD Biosciences (San Jose, CA, USA). The cells were analyzed using FACS Calibur flow cytometer (BD biosciences) to measure percentages of CD13-positive, CD90 -positive, CD146 -positive, and CD34-negative cells. Acquired data were analyzed by Cell-Quest Pro software (BD biosciences).

Alizarin red S staining, Alcian Blue staining, and Oil red O staining of hPDLSCs

Acquired hPDLSCs were cultured in 24-well plates with α-MEM containing 10% FBS at an initial density of 4.0×10^4 cells/well until 50 ~ 60% confluence was reached. Then, culture medium was changed to StemPro Osteogenic, StemPro Chondrogenic, and StemPro Adipogenic differentiation medium (Gibco BRL) to confirm multi-lineage differentiation potential with the appropriate supplements. After 7 days of post-osteogenic culture, 2% Alizarin red S (Sigma-Aldrich) staining was done at pH 4.2 to detect calcium deposits, which are indicators of osteogenic differentiation. After 14 days of post-chondrogenic culture, the cells were stained with 1% Alcian Blue (Sigma-Aldrich) to detect proteoglycans, which are

indicators of chondrogenic differentiation. After 21 days of post-adipogenic culture, fat vacuoles, indicators of adipogenic differentiation, were detected with 0.3% Oil Red O dye (Sigma-Aldrich) staining. All stained cells were visualized under an inverted light microscope (Olympus U-SPT; Olympus, Tokyo, Japan).

CCK-8 assay

Effects of calcitonin on cell proliferation and cytotoxicity were measured with the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Tech Inc., Rockville, MD, USA). WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitro-phenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] produces a water-soluble formazan dye, and the amount of the dye generated in cells is directly proportional to the number of living cells. For the CCK-8 assay, hPDLSCs (3.0×10^3 cells/well) were seeded in 96-well plates and cultured for 48 hours. Various concentrations (0 nM, 0.1 nM, 1 nM, 10 nM, 100 nM) of sCT were added and incubated in 5% CO_2 at 37℃ for 24 and 48 hours. 10 μl of CCK-8 dye solution were then added to each well of culture plate, and the cells were incubated in 5% CO_2 at 37℃ for 4 hours. The reaction products were quantified using an ELISA plate reader at 450 nM (650 nM as reference).

Each condition was prepared in triplicate, and the values are expressed as optical densities (OD).

Wound healing assay

Effects of calcitonin on hPDLSCs migration were evaluated with wound healing assay. Human PDLSCs were seeded in six-well plates with complete medium. After cell monolayers were grown to 90% confluence, they were wounded by a 1 mm plastic tip. Wounded monolayers were then washed twice with medium to remove cell debris and incubated with various concentrations (0, 0.1, 1, 10, 100 nM) of sCT. Cell migration into the wound surface was monitored by microscope at 0, 6, 12, and 24 hours. Migration was quantified by counting the number of migrating cells from the wound edge within the wound area.

ALP staining and Alizarin red S staining

The hPDLSCs (4.0×10^4 cells/well) were seeded in 24-well plates with α-MEM containing 10% FBS until 50 ~ 60% confluence was reached. To evaluate the effects of calcitonin on alkaline phosphatase activity and osteogenesis, the hPDLSCs were cultured in osteogenic differentiation medium containing 50 μg/mL ascorbic acid, 10 mM β-glycerophosphate, and 100 nM dexamethasone (Sigma-Aldrich) with various concentrations (0 nM, 0.1 nM, 1 nM, 10 nM, 100 nM) of sCT.

At day 7, ALP staining was done. Cells washed with PBS were fixed for 30 seconds with fixation solution made with 37% formalin from formaldehyde solution (DUKSAN, Ansan, Korea), citrate (Sigma-Aldrich), and acetone (Junsei Chemical Co. Ltd, Tokyo, Japan). After fixation solution was washed with distilled water, the cells were soaked in Alkaline-Dye mixture consisting of FRN-Alkaline solution, Naphthol AS-BI Alkaline, and sodium nitrite solution (Sigma-Aldrich) diluted in distilled water for 15 minutes for staining. A photograph was taken after the stained cells were washed with distilled water and dried.

At day 7 and 14, Alizarin red S staining was done as follows. First, cells were washed with PBS, fixed with ice-cold 70% ethanol, and left in room temperature for 10 minutes. After washing with PBS, 40 mM Alizarin red S (pH 4.2) solution was added to the wells for 5 minutes. Finally, Alizarin red S solution was washed with distilled water, then a photograph was taken.

RNA preparation and quantitative real-time polymerase chain reaction analysis

To evaluate gene expression levels in calcitonin-induced differentiated hPDLSCs, 1.0×10^6 cells were seeded in a 60mm culture dish and cultured with five concentrations (0, 0.1, 1, 10, 100 nM) of sCT for 7 and 14 days under differentiation induction conditions. Total RNA was prepared using an RNeasy

Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA concentration was measured using a Nanodrop ND2000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA). From 2 ㎍ of total RNA, cDNA was synthesized using reverse transcriptase (Superscript II Preamplification System; Invitrogen, Carlsbad, CA, USA). Real-time polymerase chain reaction (PCR) was performed with SYBR Green PCR Master Mix (ABI Prism 7500 sequence detection system; Applied Biosystems, Foster City, CA, USA) and a Real-Time PCR System 7500 (Applied Biosystems). The reaction protocol was set as 15 seconds at 95℃ for denaturation and 60 seconds at 60℃ for amplification. These stages were repeated for 40 cycles. All reactions were performed in triplicate and normalized to the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT). The cycle threshold values were calculated, and the expression levels of alkaline phosphatase (ALP), type I collagen (Col1), bone sialoprotein (BSP), Runt-related transcription factor-2 (Runx2), osteopontin (OPN), and osteocalcin (OCN) were evaluated and described as a histogram. The specific primer sets used for this analysis are listed in Table 1.

Statistical analysis

All assays were performed at least three times, and all data are expressed as mean ± standard deviation. Normal distribution with equal variance was analyzed using one-way analysis of variance (ANOVA) with a Tukey's procedure. p-values of less than 0.05 were considered statistically significant ($p < 0.05$).

Results

Characterization of hPDLSCs

After primary culture of hPDLSCs from extracted third molars (Fig. 1A, B), flow cytometric analysis was performed for characterization of hPDLSCs using various stem cell markers including CD13, CD34, CD90, and CD146. Among them, CD13, CD90, and CD146 were known as mesenchymal stem cell-specific markers, while CD34 was known as hematopoietic stem cell-specific marker. The percentages of positive cells were calculated according to the relative intensities of antibody-binding cells. FACS showed that approximately 98.66% of CD13, 99.94% of CD90, 95.68% of CD146, and 5.04% of CD34 were expressed, and this result confirmed that cultured hPDLSCs were of mesenchymal stem cell origin (Fig. 2).

Multipotency of cultured hPDLSCs

The multi-lineage differentiation potential of hPDLSCs *in vitro* was also verified after incubation with osteogenic, chondrogenic, and adipogenic induction medium. Under light microscope, remarkable Alizarin red S-stained mineral deposits were formed after 7 days of osteogenic culture, Alcian Blue-stained proteoglycan nodules were prominent after 14 days of

chondrogenic culture, and Oil Red O-positive lipid vacuoles were detected after 21 days of adipogenic culture (Fig. 3A, B, C).

Effects of calcitonin on proliferation of hPDLSCs *in vitro*

The cell proliferation was monitored and analyzed by CCK-8 assay after growth in medium containing 0, 0.1, 1, 10, 100 nM of sCT at 24 and 48 hours. Compared to the control (0 nM of sCT), statistically significant increase of hPDLSCs proliferation was detected when hPDLSCs was cultured with 1 nM of sCT after 48 hours, 0.1 nM and 10 nM of sCT after 24 hours ($p < 0.05$). The presence of cytotoxicity and dose-dependent inhibitory effects were not detected significantly, although 100 nM of sCT after 24 and 48 hours showed decrease in proliferation ($p > 0.05$) (Fig. 4A, B).

Effects of calcitonin on migration of hPDLSCs *in vitro*

Wound healing assay was used to evaluate whether calcitonin affects cell migration of hPDLSCs. After 1 mm-wound formation and culture with 0 nM (control), 0.1, 1, 10, 100 nM of sCT, observation under microscope at 0, 6, 12, 24 hours were done to count the number of migrating cells from the wound

edge within the wound area (Fig. 5A). 1 nM sCT treated cells showed about 2-fold increased migration into the wound area at all time point, whereas other concentration of sCT treated group did not show significant difference compared to control group (Fig. 5B). These results suggest that treatment with 1 nM sCT efficiently accelerates the migratory activity of hPDLSCs.

Effects of calcitonin on osteogenic differentiation of hPDLSCs *in vitro*

First, calcitonin-induced osteogenesis was examined with ALP staining and Alizarin red S staining. 1 nM sCT treated hPDLSCs showed remarkable increase in ALP activity after 7 days of ALP staining with osteogenic induction medium (Fig. 6A). Alizarin red S staining also confirmed that 1 nM sCT treated hPDLSCs showed higher osteogenic tendency both at 7 and 14 days compared to other concentration of sCT (Fig. 6B).

Effects of calcitonin on osteogenic differentiation markers expression *in vitro*

Using real-time PCR, relative mRNA expression levels of ALP, Col1, BSP, Runx2, OPN, and OCN of hPDLSCs were quantified after calcitonin induced osteogenesis at 7 and 14

days. 1 nM sCT treated hPDLSCs showed significant increase in expression of Col1 (2.2-fold) and OPN (1.8-fold) compared to control group at day 7 (Fig. 7A). At day 14, expression levels of ALP (2.0-fold), Col1 (3.9-fold), BSP (7.9-fold), and OPN (2.8-fold) were increased significantly in 1 nM sCT treated group compared to control, respectively (Fig. 7B). BSP, which did not reveal significant difference at day 7, increased to the greatest extent at day 14. OCN showed decreased expression (0.6-fold) at day 7, but expression level increased (1.2-fold) at day 14. One of the early osteogenic markers, Runx2, showed significant increase in expression level (1.8-fold) when treated with 100 nM sCT at day 14 (Fig. 7A, 7B).

Discussion

This *in vitro* study investigated the calcitonin-induced effects on proliferation, migration, and differentiation of hPDLSCs to evaluate the usefulness of calcitonin in stem cell therapy for regenerative medicine. First, PDLSCs were isolated from the extracted human third molars and confirmed of its mesenchymal stem cell-like properties by FACS analysis and multi-lineage differentiation with Alizarin red S staining, Alcian blue staining and Oil red O staining.

Under 5 groups of nanomolar concentrations of calcitonin (0 nM, 0.1 nM, 1 nM, 10 nM, 100 nM), the effects of calcitonin on proliferation and cytotoxicity were evaluated with CCK-8 assay. Low concentration of sCT (1 nM after 48 hours, 0.1 nM and 10 nM after 24 hours) showed increase in proliferation of hPDLSCs (p <0.05), and high concentration of sCT (100 nM after 24 and 48 hours) suppressed proliferation (p >0.05). These results were similar with calcitonin treatment on huAFMSCs, but dose-dependent inhibitory effects on proliferation were not as clear as huAFMSCs[17]. According to previous studies, calcitonin controls the migration of osteoclast precursors[18-20], and low concentration (1 nM) of sCT increased motility of hPDLSCs by 2-fold in wound healing assay.

Calcitonin induced effects on osteogenic differentiation of hPDLSCs were identified by Alizarin red S staining, ALP staining and real-time PCR. ALP staining was done at day 7 to evaluate the ALP activity, and Alizarin red S staining was done at day 7 and 14 to determine changes in mineralization. As described above, most effective osteogenesis was observed with 1 nM sCT treatment group on both 7 and 14 days. Higher concentration did not increase the mineralization efficacy, and there was no dose-dependent effect.

These results were consistent with the real-time PCR data. ALP, Col1, BSP, Runx2, OPN, and OCN are known to be key markers involved in osteogenic lineage[21]. Among them, ALP, type I collagen, and Runx2 are relatively early transcription factors, and osteocalcin is late transcription factor. Osteopontin showed two peak elevated expression during proliferation and later stages of differentiation[22]. In our study, when treated with 1 nM of sCT, Col1 and OPN mRNA expressions significantly increased first at day 7. ALP, Col1, BSP, OPN, and OCN mRNA expression were all increased at day 14. Meanwhile, Runx2 showed significant increased expression level with 100 nM sCT at day 14, but not with 1 nM sCT. Taken together, 1 nM sCT treatment promotes the osteogenic differentiation of hPDLSCs, and Runx2 might be regulated via different mechanism. Furthermore, the result that low concentration of sCT induced

osteogenic differentiation, cell migration, and cell proliferation all together is quite interesting because cell proliferation and differentiation progress in the opposite way usually.

Previous studies reported that calcitonin mediates cyclic adenosine monophosphate (cAMP) / protein kinase A (PKA) signaling pathway or protein kinase C (PKC) pathway in a tissue-dependent manner, and cAMP / PKA signaling is known to promote osteogenesis on various stem cells[20,23,24]. Morabito *et al*. reported that low concentration (1 nM) of sCT increased osteogenesis in huAFMSCs via cAMP / PKA signaling[17]. On the other hand, different expression tendency of Runx2 in sCT treated hPDLSCs may be related to PKC activity, which stabilizes Msx2, leading to Runx2 inhibition, and finally suppressing osteogenic differentiation with promoting proliferation of osteoprogenitor cells[25,26,27]. However, little is known of calcitonin induced signaling pathway due to the complexity and diversity of CT/CTR system and CT family factor[28].

Some studies found that CTR mainly acts in times of high calcium stress condition and the rate of bone turnover such as growth, pregnancy, and lactation[4,23,29]. Calcitonin induced action on hypothalamus is also supposed to involve in bone metabolism[30]. However, the role of calcitonin in mammalian physiology and *in vivo* model has been questioned due to its

ambiguous features that patients who had thyroidectomy or medullary thyroid carcinoma did not show significant bone pathophysiology, and mice lacking *Calca* gene, which encodes CT, rather displayed high bone mass[2,23,31].

Although calcitonin is not a first choice of drug for osteoporosis at present, side effects of other anti-resorptive agents such as bisphosphonates have risen. Salmon calcitonin has been the drug of choice in the treatment of osteoporosis and hypercalcemia due to its higher potency than mammalian calcitonin[2,3,7,32]. There has been a debate of carcinogenesis with the use of calcitonin, but Wells *et al.* reported a review and meta-analysis that showed little evidence and weak association between salmon calcitonin and cancer[33].

Multipotent dental stem cells have been studied for use in the tissue regeneration therapy[34,35]. Considering effects of calcitonin on osteogenic differentiation of hPDLSCs, further studies of *in vivo* model and involved signaling pathways are necessary for clinical application.

Conclusion

Application of mesenchymal stem cells in regenerative medicine is getting more attention, especially the use of dental stem cells in regenerating dental tissue and osseous defects. Periodontal ligament stem cells have multi-lineage differentiation potential, and regulation of PDLSCs biology has been widely researched with various naturally existing and/or artificially synthesized molecules. For the first time, this study demonstrated that low nanomolar concentration (1 nM) of calcitonin induces proliferation of hPDLSCs without cytotoxicity. Also, it accelerates migration of hPDLSCs and promotes osteogenic differentiation as well. Taken together, the results of this *in vitro* study support the validity of further calcitonin use with dental stem cells in regenerative medicine.

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A. B.

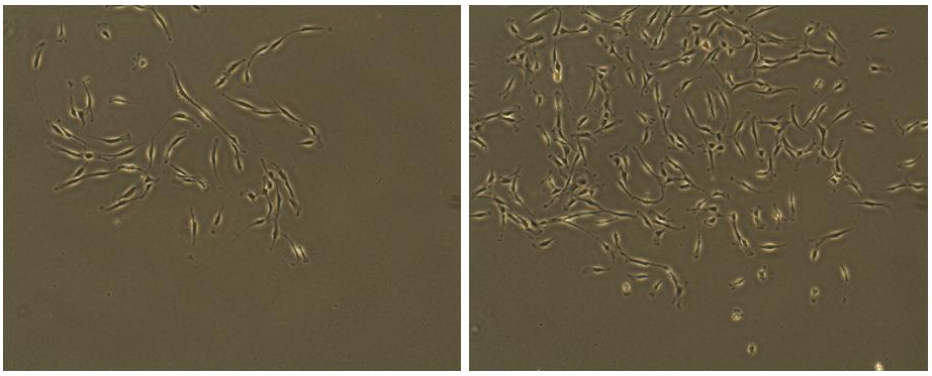


Fig. 1. Primary hPDLSCs cultured from extracted third molars.

A. Spindle shaped cultured hPDLSCs were observed at 5 days of passage 0 under light microscope.

B. Normal proliferation of cultured hPDLSCs was observed at 7 days of passage 0 under light microscope.

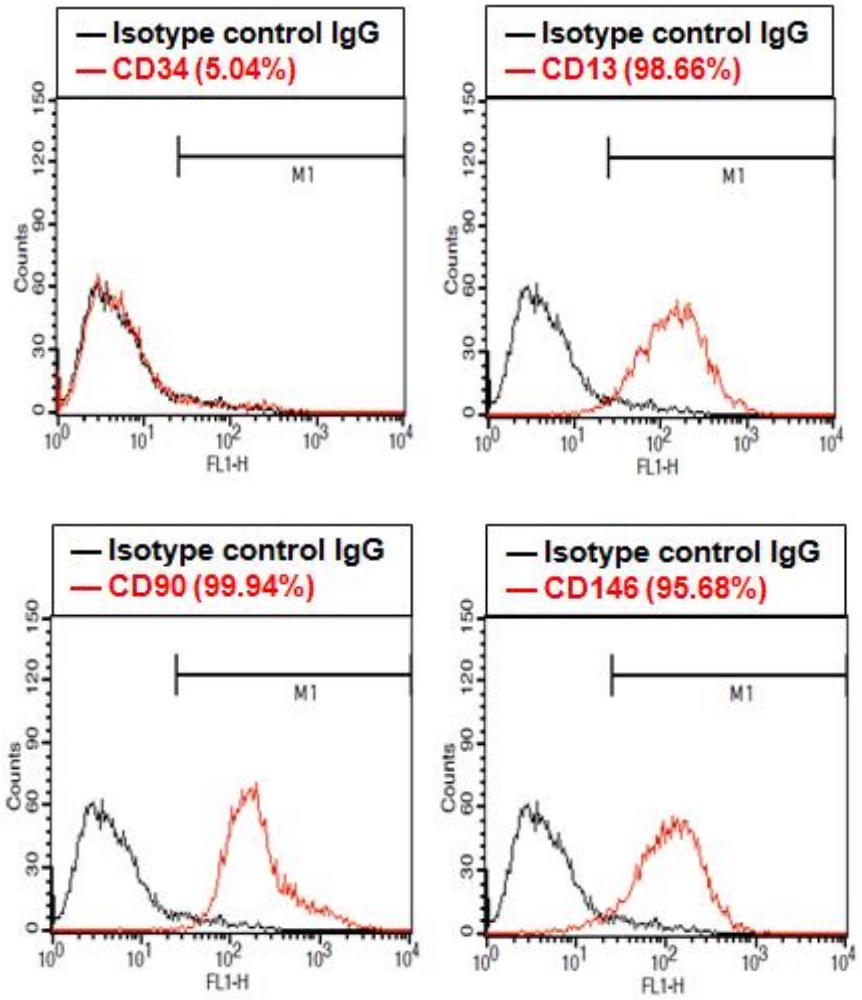


Fig. 2. Characterization of hPDLSCs by FACS.

Flow cytometry analysis (FACS) of hPDLSCs using mesenchymal stem cell markers such as CD13, CD34, CD90, and CD146. To analyze each population of CD13−, CD34−, CD90−, and CD146−positive cells, the percentages of the cells to the right of the M1 gate were measured.

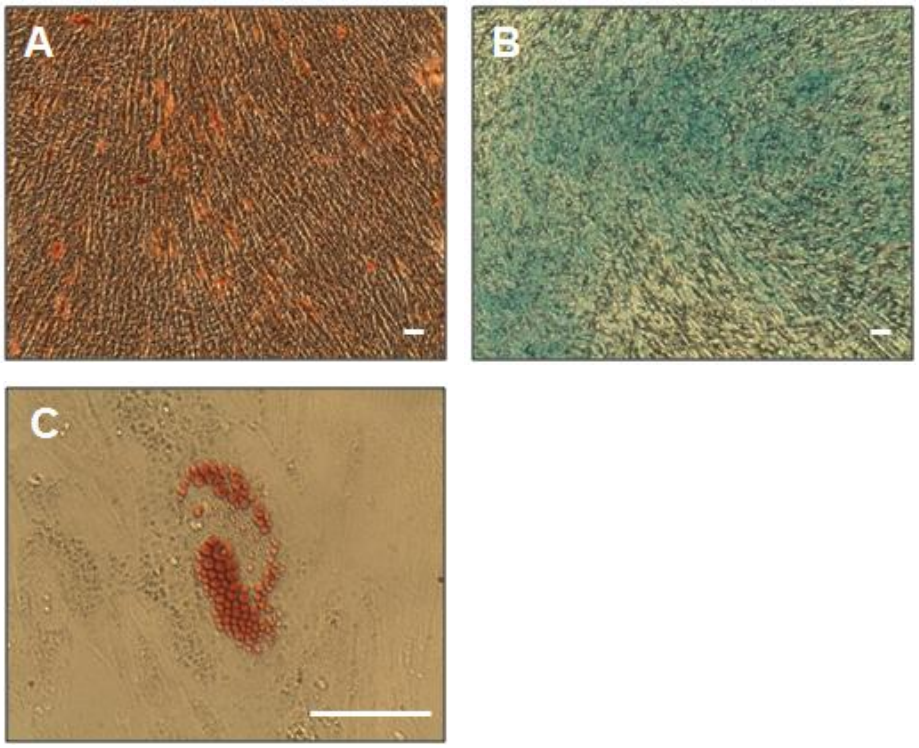


Fig. 3. Multi-lineage differentiation of hPDLSCs.

The multi-lineage differentiation potential of hPDLSCs *in vitro* was investigated by Alizarin red S staining after incubation with osteogenic medium, Alcian Blue staining after incubation with chondrogenic medium, and Oil red O staining after incubation with adipogenic medium. (A) Under light microscope, remarkable Alizarin red S-stained mineral deposits were formed after 7 days of osteogenic culture. (B) Alcian Blue-stained proteoglycan nodules were prominent after 14 days of chondrogenic culture. (C) Oil Red O-positive lipid vacuoles were detected after 21 days of adipogenic culture. Scale bar = 40 μm.

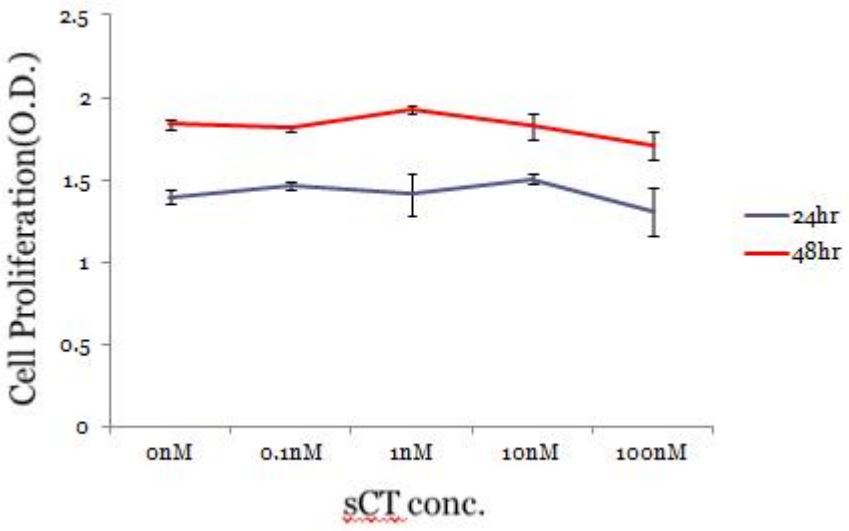
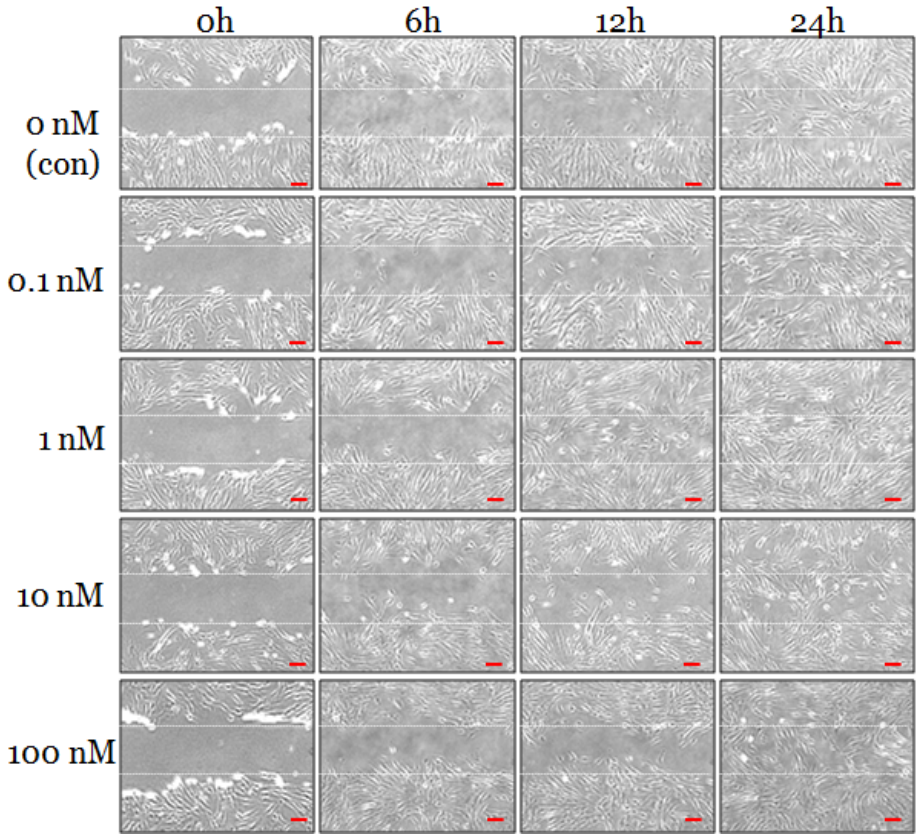


Fig. 4. Effects of calcitonin on proliferation of hPDLSCs *in vitro*.

For CCK-8 assay, hPDLSCs were cultured in 96-well plates for 48 hours. After cell attachment, proliferation of hPDLSCs were compared after treatment with 0, 0.1, 1, 10, 100 nM of salmon calcitonin (sCT) for 24 and 48 hours. Increased proliferation was detected with 1 nM of sCT after 48 hours, 0.1 nM and 10 nM of sCT after 24 hours compared to the control (p <0.05). No significant dose-dependent cytotoxicity was observed.

A.


0h
6h
12h
24h
0 nM
(con)
0.1 nM
1 nM
10 nM
100 nM

B.

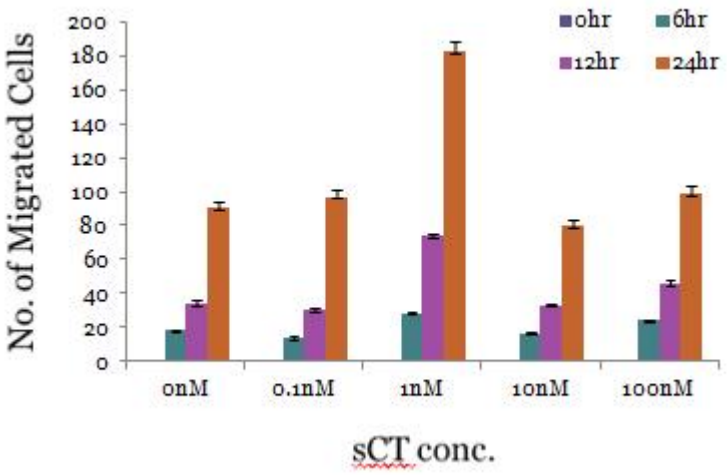
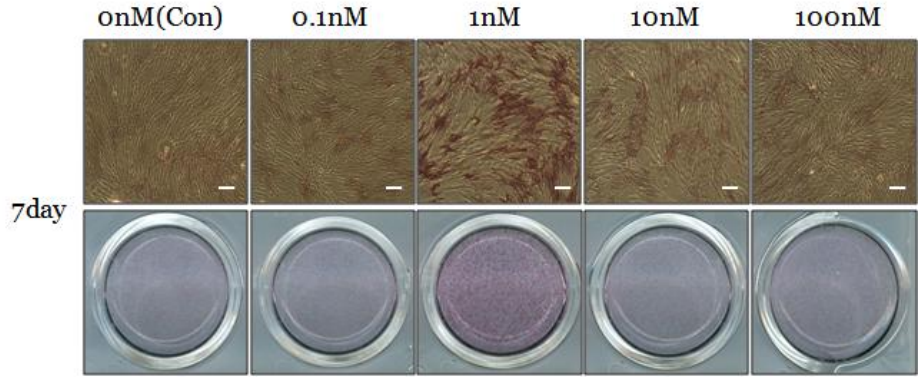

No. of Migrated Cells
200
180
160
140
120
100
80
60
40
20
0
0hr
6hr
12hr
24hr
0nM
0.1nM
1nM
10nM
100nM
sCT conc.

Fig. 5. Effects of calcitonin migration of hPDLSCs *in vitro*.

Wound healing assay was performed to examine the effects of sCT on migration of hPDLSCs *in vitro*. After 1mm-wound formation in cell monolayers with 90%-confluency, treatments with 0 nM (control), 0.1 nM, 1 nM, 10 nM, 100 nM of sCT were done. (A) Observation under microscope was performed at 0, 6, 12, 24 hours. (B) 1 nM sCT treated cells showed about 2-fold increased migration into the wound area at all time point, whereas other concentration of sCT treated group did not show significant difference compared to control group. Scale bar = 40 μm.

A.


0nM(Con)
0.1nM
1nM
10nM
100nM
7day

B.

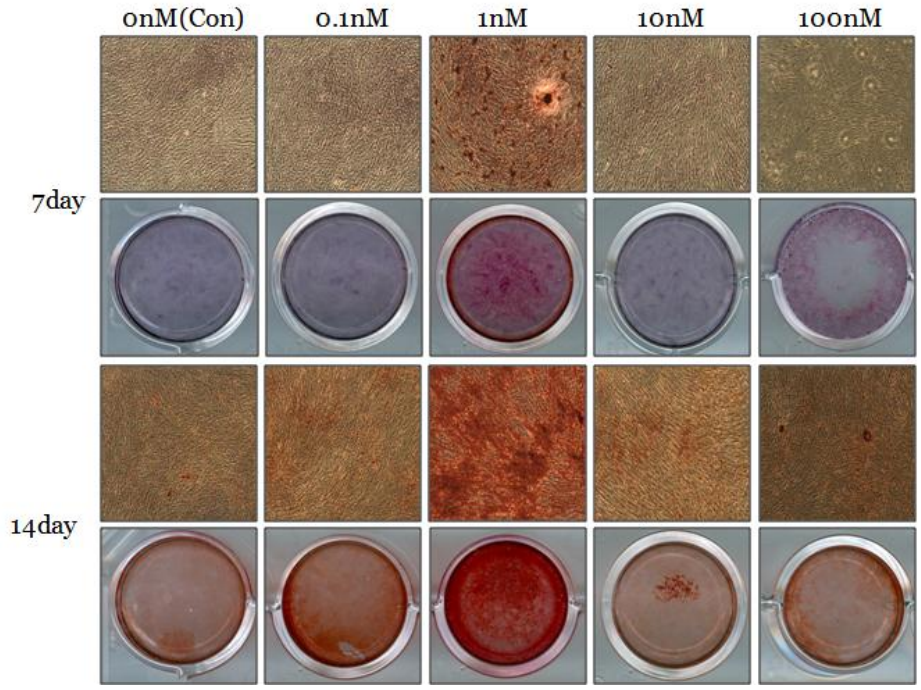
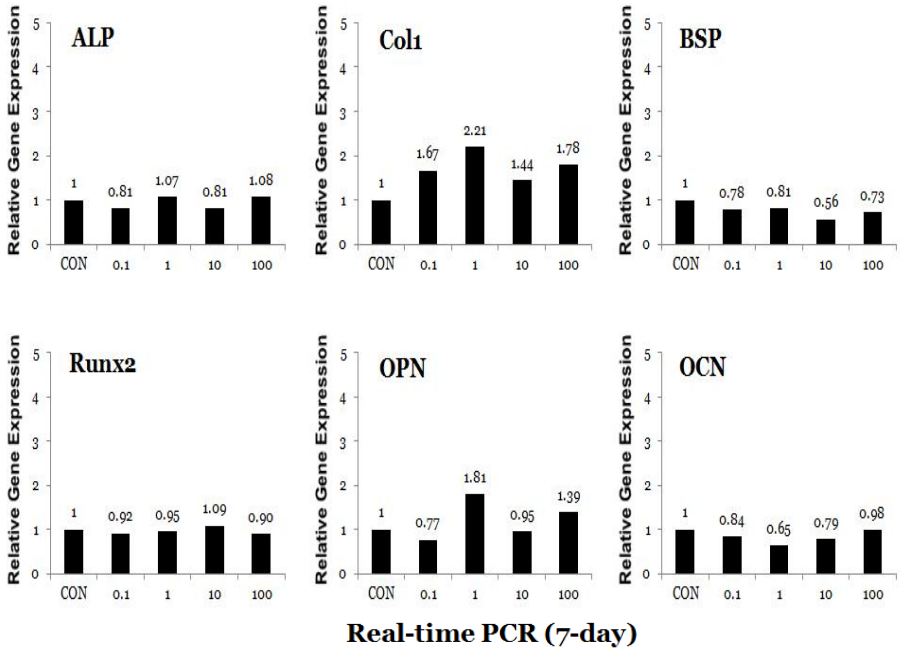

0nM(Con)
0.1nM
1nM
10nM
100nM
7day
14day

Fig. 6. Effects of calcitonin on osteogenic differentiation of hPDLSCs *in vitro*.

hPDLSCs were cultured in osteogenic medium with 0 nM (control), 0.1 nM, 1 nM, 10 nM, 100 nM treatment of sCT, and ALP staining and Alizarin red S staining were done. (A) 1 nM sCT treated hPDLSCs showed remarkable increase in ALP activity after 7 days of ALP staining with osteogenic induction medium. (B) Alizarin red S staining showed higher mineralization tendency of 1 nM sCT treated hPDLSCs both at 7 and 14 days when compared to other concentrations of sCT. Scale bar = 40 μm.

A.


ALP
Col1
BSP
Runx2
OPN
OCN
Relative Gene Expression
CON
0.1
1
10
100
1 0.81 1.07 0.81 1.08
1 1.67 2.21 1.44 1.78
1 0.78 0.81 0.56 0.73
1 0.92 0.95 1.09 0.90
1 0.77 1.81 0.95 1.39
1 0.84 0.65 0.79 0.98
Real-time PCR (7-day)

B.

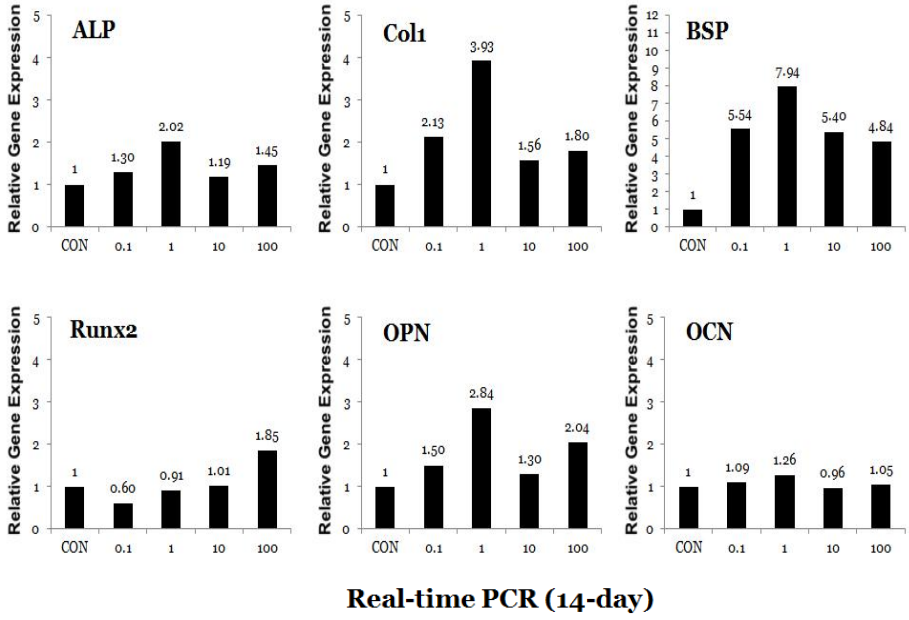

ALP
Col1
BSP
Runx2
OPN
OCN
Relative Gene Expression
CON
0.1
1
10
100
1 1.30 2.02 1.19 1.45
1 2.13 3.93 1.56 1.80
1 5.54 7.94 5.40 4.84
1 0.60 0.91 1.01 1.85
1 1.50 2.84 1.30 2.04
1 1.09 1.26 0.96 1.05
Real-time PCR (14-day)

Fig. 7. The mRNA expression of osteogenic differentiation markers of calcitonin-treated hPDLSCs *in vitro*.

Expression of osteogenesis-related genes were analyzed using quantitative real-time polymerase chain reaction. ALP, Col1, BSP, Runx2, OPN, and OCN of hPDLSCs were investigated after calcitonin induced osteogenesis at day 7 (A) and at day 14 (B).

Table 1. Primer sequences used in the Real-time PCR.

Gene	GenBank No.	Sequences
ALP	NM_007431	5'-CCAACTCTTTTGTGCCAGAGA-3' 5'-GGCTACATTGGTGTTGAGCTTTT-3'
Col1	NM_007742	5'-GCTCCTCTTAGGGGCCACT-3' 5'-CCACGTCTCACCATTGGGG-3'
BSP	L09555	5'-AACTTTTATGTCCCCCGTTGA-3' 5'-TGGACTGGAAACCGTTTCAGA-3'
Runx2	NM_001024630.3	5'-CACCATGTCAGCAAAACTTCTT-3' 5'-ACCTTTGCTGGACTCTGCAC-3'
OPN	J04765	5'-TGAAACGAGTCAGCTGGATG-3' 5'-TGAAATTCATGGCTGTGGAA-3'
OCN	AL135927	5'-TGAGAGCCCTCACACTCCTC-3' 5'-ACCTTTGCTGGACTCTGCAC-3'
HPRT	NM_000194	5'-GGCTATAAGTTCTTTGCTGACCTG-3' 5'-CCACAGGGACTAGAACACCTGCTA-3'

국문초록

칼시토닌이 사람 치주인대유래 치아줄기세포에 미치는 효과

김 수 호

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(지도교수 : 정 필 훈)

목적

칼시토닌은 신체에서 칼슘대사에 관여하는 호르몬으로 혈중 칼슘농도를 낮추고 골 조직에 칼슘 이온을 저장하는 역할을 한다. 치아줄기세포는 다계통 분화가 가능하기에 재생의학분야에서 계속 연구되어 왔다. 이 연구의 목적은 칼시토닌이 사람 치주인대유래 줄기세포에 미치는 영향을 분석하기 위함이다.

재료 및 방법

발치한 제 3 대구치에서 치주인대유래 줄기세포를 분리하여 일차 배양 후 유세포분석(FACS), Alizarin red S 염색, Alcian Blue 염색,

Oil red O 염색을 통해 다계통 분화가 가능한 줄기세포 여부를 확인하였다. 칼시토닌이 사람 치주인대유래 치아줄기세포의 증식, 이동, 분화에 미치는 영향을 확인하기 위해 CCK-8 assay, Wound healing assay, ALP 염색, Alizarin red S 염색, 그리고 Real-time polymerase chain reaction (Real-time PCR)을 시행하였다.

결과

배양된 사람 치주인대유래 줄기세포가 중간엽 유래 줄기세포의 특징을 갖고 있고, 골조직, 연골조직, 지방조직으로 분화가 가능한 것을 확인하였다. 24 시간과 48 시간 관찰시 유의한 세포독성이 관찰되지 않았으며, 1 nM 의 칼시토닌 처리시 48 시간째 대조군에 비해 세포 증식이 제일 증가하였다. 동일 농도 칼시토닌 처리시 세포이동성이 제일 증가하였고, 골분화 관련 인자 발현도 가장 증가하였다.

결론

본 연구에서 1 nM 농도의 칼시토닌이 사람 치주인대유래 치아줄기세포의 골분화 및 세포 증식과 이동을 모두 촉진하는 것을 확인하였다. 이 결과는 칼시토닌이 골다공증 및 고칼슘혈증의 치료뿐만 아니라 재생의학에서도 활용될 수 있는 근거를 뒷받침해준다.

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주요어 : 칼시토닌, 치아줄기세포, 사람 치주인대, 골 재생, 재생의학

학 번 : 2010-23755