Journal of
PERIODONTAL RESEARCH

J Periodont Res 2010; 45: 504–511

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2009.01265.x

Activation of cannabinoid receptor CB2 regulates osteogenic and osteoclastogenic gene expression in human periodontal ligament cells

H. Qian[1], Y. Zhao[2], Y. Peng[3], C. Han[1], S. Li[1], N. Huo[1], Y. Ding[1], Y. Duan[1], L. Xiong[4], H. Sang[4]

[1]Department of Orthodontics, School of Stomatology, Fourth Military Medical University, Xi'an, Shaanxi, China, [2]Department of Medical Microbiology and Parasitology, Fourth Military Medical University, Xi'an, Shaanxi, China, [3]Department of Ortheopedics, Xijing Hospital, Fourth Military Medical University, Xi'an, Shaanxi, China and [4]Department of Anesthesiology, Xijing Hospital, Fourth Military Medical University, Xi'an, Shaanxi, China

Qian H, Zhao Y, Peng Y, Han C, Li S, Huo N, Ding Y, Duan Y, Xiong L, Sang H. Activation of cannabinoid receptor CB2 regulates osteogenic and osteoclastogenic gene expression in human periodontal ligament cells. J Periodont Res 2010; 45: 504–511. © 2010 John Wiley & Sons A/S

Background and Objective: Cannabinoid receptor CB2, expressed in osteoblasts and osteoclasts, plays a crucial role in the regulation of bone metabolism. Since periodontal ligament (PDL) cells can differentiate into osteoblasts, this study was undertaken to investigate CB2 expression and the effect of CB2 activation on osteogenic differentiation of PDL cells.

Material and Methods: Human PDL (hPDL) cells were obtained from extracted teeth of periodontally healthy subjects. Expression of CB2 was observed in hPDL cells by RT-PCR, Western blotting and immunofluorescence assay. Then hPDL cells were treated with a CB2-specific agonist, HU-308 (10^{-7} M), for 12, 24, 48 or 72 h. The mRNA expressions of osteogenic genes, such as runt-related transcription factor 2 (*Runx2*), bone sialoprotein (*BSP*), osteopontin (*OPN*), alkaline phosphatase (*ALP*), osteocalcin (*OC*) and collagen type I (*COL I*), and osteoclastogenic genes, including osteoprotegerin (*OPG*) and receptor activator of NF-κB ligand (*RANKL*), were examined using quantitative real-time PCR analysis. A mineralization assay was performed in hPDL cells in mineralization conditions with or without HU-308.

Results: Expression of CB2 mRNA and protein was detected in hPDL cells. HU-308 enhanced the mRNA levels of the above osteogenic genes. Expression of the *OPG* gene was up-regulated, whereas *RANKL* gene expression was down-regulated, contributing to the elevated *OPG*/*RANKL* ratio. Accelerated mineralization was observed in hPDL cells in mineralization conditions with HU-308.

Conclusion: Our findings demonstrate that activation of CB2 is able to enhance osteogenic differentiation of hPDL cells and potentially create a favorable osteogenic microenvironment. This implies that CB2 might play an important role in alveolar bone metabolism.

Hanfei Sang and Lize Xiong, Department of Anesthesiology, Xijing Hospital, Fourth Military Medical University, 15 West Changle Road, Xi'an, Shaanxi 710032, China
Tel: +86 29 84771262
Fax: +86 29 84771262
e-mail: qianred212@gmail.com (H. Sang), stomed@fmmu.edu.cn (L. Xiong)
H. Qian, Y. Zhao and Y. Peng contributed equally to this work.

Key words: human periodontal ligament cell; cannabinoid receptor; osteogenic differentiation; alveolar bone metabolism

Accepted for publication November 3, 2009

The periodontium is composed of the gingival soft tissue, the cementum covering the tooth root, the alveolar bone and the periodontal ligament (PDL; 1). The PDL is a non-mineralized connective tissue located between the hard tissues of cementum and alveolar bone. The PDL cells are composed of heterogeneous cell populations that exhibit osteoblast-like features and are capable of differentiating into either osteoblasts or cementoblasts (2–5). In addition, PDL cells can modulate osteoclastogenesis by expression of osteoprotegerin (*OPG*; 6) and receptor activator of nuclear factor-κB ligand (*RANKL*; 7), which are two critical osteoclastogenic genes in regulating osteoclast differentiation and function. Therefore, PDL cells are actively involved in alveolar bone metabolism. It is well known that the participation of PDL cells in alveolar bone metabolism plays a pivotal role in the remodeling of alveolar bone during orthodontic tooth movement and the regeneration of alveolar bone destroyed by periodontal diseases or trauma (8–11). It is therefore of great importance to elucidate this mechanism and to search for more effective therapeutic strategies in the remodeling and regeneration of alveolar bone.

Cannabinoids are a heterogeneous group of molecules that bind to cannabinoid receptors. They can be divided into three groups: endogenous (endocannabinoids), synthetic and phytocannabinoids (12). The presence and functions of the endocannabinoid system have been discovered in a variety of tissues, such as brain, kidney and liver (13,14). Recently, endocannabinoids and cannabinoid receptor CB2 have been identified in the skeleton. The skeleton appears to be the main system physiologically regulated by CB2 (15–18). Both osteoblasts and osteoclasts express CB2 (14,17). Animal experiments have demonstrated that CB2-deficient mice display a markedly accelerated age-related bone loss, and activation of CB2 attenuates ovariectomy-induced bone loss in mice by enhancing bone formation and restraining bone resorption (17). Clinical investigations have confirmed that the polymorphism in cannabinoid receptor CB2 gene (*CNR2*), which encodes CB2, is an important determinant in the aetiology of osteoporosis (19,20). Therefore, CB2 plays a crucial role in the regulation of bone metabolism.

Since PDL cells can differentiate into osteoblasts, we hypothesized that PDL cells might express CB2 and that activation of CB2 would influence osteogenic differentiation of PDL cells. Therefore, this study was undertaken to test the above hypothesis. As the first step, we observed CB2 expression in human PDL (hPDL) cells. Next, we treated hPDL cells with a CB2-specific agonist, HU-308, and examined the mRNA expression of several essential osteogenic and osteoclastogenic genes. Finally, mineralized nodule formation was assessed in hPDL cells in mineralization conditions with or without HU-308.

Material and methods

Cell culture

Healthy hPDL tissue was obtained from the extracted (for orthodontic reasons) premolars of two males (12 and 15 years old) and one female (13 years old). All patients gave written informed consent before tooth extraction. Ethical approval had been obtained from the Ethics Committee of Fourth Military Medical University, Xi'an, Shaanxi, China. As described previously (21), the PDL tissue attached to the mid-third of the root surface was scraped off, cut into small pieces and placed into tissue culture flasks. The explants were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma, St Louis, MO, USA) containing 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 100 U/mL of penicillin and 100 μg/mL of streptomycin at 37°C in a humidified atmosphere of air enriched with 5% CO_2. When the hPDL cells growing from the tissue fragments reached confluence, the cell layer was rinsed with phosphate-buffered saline (PBS) and the cells were released with 0.25% trypsin–0.1% EDTA solution. The hPDL cells used in this study were from passage 3–5. The mouse MC3T3 E1 osteoblastic cell line, known to express CB2 (17), was obtained from Department of Biochemistry, Fourth Military Medical University, Xi'an, Shaanxi, China. The cells were cultured in α-modified Eagle's minimal essential medium (α-MEM; Sigma, St Louis, MO, USA) containing 10% FBS, 100 U/mL of penicillin and 100 μg/mL of streptomycin at 37°C in a humidified atmosphere of air enriched with 5% CO_2.

Extraction of RNA and RT-PCR analysis

The total RNA of hPDL cells and MC3T3 E1 cells was extracted using RNAiso plus Reagent (TaKaRa Biotechnology Co. Ltd, Dalian, China) according to the manufacturer's instructions. Samples of total RNA were used to perform first strand cDNA synthesis using the PrimeScript™RT Reagent Kit (TaKaRa Biotechnology Co. Ltd). One-tenth of cDNA generated was used as a template for PCR by Amplification Kit (TaKaRa Biotechnology Co. Ltd). The primer sequences and product sizes of each gene [*CB2* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)] are shown in Table 1. The cycling conditions for each gene were as follows: *CB2*, 94°C for 3 min, followed by 94°C for 30 s, 65°C for 30 s and 72°C for 40 s for 35 cycles, and 72°C for 5 min; and *GAPDH*, 94°C for 3 min, followed by 94°C for 30 s, 60°C for 30 s and 72°C for 30 s for 35 cycles, and 72°C for 5 min. The PCR products were separated on 2% agarose gels by electrophoresis and photographed under ultraviolet excitation after ethidium bromide staining.

Western blotting analysis

The hPDL cells and MC3T3 E1 cells were collected and rinsed twice with cold PBS. Then the cells were solubilized with 1× lysis buffer (pH 8.0) that consisted of 50 mM Tris–HCl, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 0.5 mM phenylmethanesulfonyl fluoride (PMSF), 10 μg/mL of leupeptin and 1 μg/mL of aprotinin. The supernatant was

Table 1. Sequence of target gene primers

Target gene	Primer sequence (5′–3′)	Length (bp)	Reference
CB2	Forward: CGCCGGAAGCCCTCATACC Reverse: CCTCATTCGGGCCATTCCTG	523	(22)
GAPDH	Forward: GCACCGTCAAGGCTGAGAAC Reverse: TGGTGAAGACGCCAGTGGA	138	This study
Runx2	Forward: CACTGGCGCTGCAACAAGA Reverse: CATTCCGGAGCTCAGCAGAATAA	127	This study
BSP	Forward: CTGGCACAGGGTATACAGGGTTAG Reverse: ACTGGTGCCGTTTATGCCTTG	182	This study
OPN	Forward: ACACATATGATGGCCGAGGTGA Reverse:TGTGAGGTGATGTCCTCGTCTGTAG	115	This study
ALP	Forward: GGACCATTCCCACGTCTTCAC Reverse: CCTTGTAGCCAGGCCCATTG	137	This study
OC	Forward: CGGTGCAGAGTCCAGCAAAG Reverse: TACAGGTAGCGCCTGGGTCTCT	84	This study
COL I	Forward: CTGCTGGACGTCCTGGTGAA Reverse: ACGCTGTCCAGCAATACCTTGAG	131	This study
OPG	Forward: AGCTGCAGTACGTCAAGCAGGA Reverse: TTTGCAAACTGTATTTCGCTCTGG	164	This study
RANKL	Forward: ATCGTTGGATCACAGCACATCAG Reverse:GGATGTCGGTGGCATTAATAGTGAG	127	This study

Abbreviations: *CB2*, cannabinoid receptor 2; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *Runx2*, runt-related transcription factor 2; *BSP*, bone sialoprotein; *OPN*, osteopontin; *ALP*, alkaline phosphatase; *OC*; osteocalcin; *COL I*, collagen type I; *OPG*, osteoprotegerin; and *RANKL*, receptor activator of nuclear factor-κB ligand.

obtained by centrifugation at 12,000*g* for 15 min at 4°C. Samples containing equal amounts of protein were electrophoresed in 5% stacking and 12% resolving sodium dodecyl sulfate–polyacrylamide gels and were transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% (w/v) skimmed milk in Tris-buffered saline (TBS) at 37°C for 1 h and then incubated at 4°C overnight with a 1:100 dilution of primary rabbit anti-CB2 serum (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-β-actin serum, followed by incubation at 37°C for 1 h with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:1000 dilution; Santa Cruz Biotechnology). Developer and fixer processing was done at the end of the incubation.

Immunofluorescence assay

The single-cell monolayers (hPDL cells and MC3T3 E1 cells) on tissue culture coverslips inside 24-well plates were fixed in cold acetone for 10 min and permeabilized in 0.5% Triton X-100 in PBS for 5 min. After washing, the cells were blocked with normal goat serum (1:50 dilution) at 37°C for 1 h and then incubated at 4°C overnight with a 1:100 dilution of primary rabbit anti-CB2 serum (Santa Cruz Biotechnology). Normal rabbit serum was used as the negative control. Next, the cells were incubated at 37°C for 1 h with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G (1:500 dilution; Santa Cruz Biotechnology), avoiding light. Fluorescence staining of cells was observed under a fluorescence microscope (Olympus, Tokyo, Japan).

Quantitative real-time PCR analysis

The hPDL cells were seeded in six-well plates at a density of 1×10^6 cells per well and allowed to attach for 12 h. Next, the cells were silenced with serum-free DMEM overnight. They were then treated with 10^{-7} M HU-308 (Cayman Chemical, Ann Arbor, MI, USA) for 12, 24, 48 or 72 h. The untreated hPDL cells served as the control group. To quantify the mRNA expression of runt-related transcription factor 2 (*Runx2*), bone sialoprotein (*BSP*), osteopontin (*OPN*), alkaline phosphatase (*ALP*), osteocalcin (*OC*), collagen type I (*COL I*), osteoprotegerin (*OPG*) and receptor activator of NF-κB ligand (*RANKL*), we performed quantitative real-time PCR using PrimeScript™RT Reagent Kit Perfect Real-Time with a SYBR Green Reagent (TaKaRa Biotechnology Co. Ltd) in the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The primer sequences and product sizes of each gene are shown in Table 1. The cycling conditions were as follows: 95°C for 30 s, followed by 95°C for 5 s and 60°C for 30 s for 40 cycles. Reaction products of all the genes were normalized to GAPDH.

Mineralization assay

The hPDL cells were seeded in six-well plates at a density of 1×10^5 cells per well and allowed to develop to confluence. Then, the cells were cultured in osteogenic induction medium, with or without 10^{-7} M HU-308. The osteogenic induction medium consisted of DMEM supplemented with 10% FBS, 10 nM dexamethasone, 50 μg/mL of ascorbic acid and 10 mM β-glycerophosphate (23). On day 21, the samples were fixed with 4% polyoxymethylene for 0.5 h. Alizarin Red staining was performed to determine mineralization as previously reported (24). The mineralized nodules were imaged and analyzed with Leica Q-Win image analysis system (Leica, Cambridge, UK).

Statistical analysis

All data are presented as means ± SD from three independent experiments. The statistical differences between the groups were determined using Bonferroni's modification of Student's paired *t*-test, and *p*-values < 0.05 were considered to be statistically significant.

Results

Expression of CB2 in hPDL cells

Expression of CB2 in hPDL cells was evaluated from mRNA to protein levels by RT-PCR, Western blotting and

immunofluorescence assay. Expression of CB2 mRNA was detected in hPDL cells using RT-PCR analysis (Fig. 1A, lane 1). Consistent with this result, Western blotting analysis detected CB2 protein expression in hPDL cells (Fig. 1B, lane 1). The MC3T3 E1 cells were used as the positive control (Fig. 1A, lane 2 and Fig. 1B, lane 2). The immunofluorescence assay was done to elucidate the expression and subcellular localization of CB2 protein. Clear green fluorescence of CB2 protein was observed in hPDL cells (Fig. 2B), whereas fairly mild green fluorescence was observed in the negative control culture, in which normal rabbit serum was used instead of primary antibody against CB2 (Fig. 2A). The MC3T3 E1 cells were used as the positive control (Fig. 2C).

Effect of HU-308 on osteogenic gene expression in hPDL cells

The effect of HU-308 on the mRNA expression of osteogenic genes, including *Runx2*, *BSP*, *OPN*, *ALP*, *OC* and *COL I*, were assessed in hPDL cells by quantitative real-time PCR analysis. The mRNA expression of *Runx2* and *OPN* increased markedly after 24 h of treatment and peaked at 72 h ($p < 0.05$; Fig. 3A,C). The *BSP* mRNA expression in the HU-308-treated group increased significantly after 48 h of treatment and then decreased slightly at 72 h, but was still higher than that in the control group ($p < 0.05$; Fig. 3B). The level of *ALP* mRNA was enhanced significantly within 72 h of treatment, with the peak level at 48 h ($p < 0.05$; Fig. 3D). *OC* mRNA expression was promoted significantly after 48 h of treatment and peaked at 72 h ($p < 0.05$, Fig. 3E). The level of *COL I* mRNA increased markedly after 24 h of treatment, with the highest level at 48 h, and then decreased slightly at 72 h ($p < 0.05$; Fig. 3F).

Effect of HU-308 on osteoclastogenic gene expression in hPDL cells

The effect of HU-308 on the mRNA expression of osteoclastogenic genes, *OPG* and *RANKL*, was investigated in hPDL cells. Quantitative real-time PCR analysis indicated that HU-308 significantly up-regulated *OPG* mRNA expression after 48 h of treatment. At 72 h, the level of *OPG* mRNA in the HU-308-treated group was slightly reduced, but still higher than that in the control group ($p < 0.05$; Fig. 4A). Conversely, HU-308 significantly down-regulated *RANKL* mRNA expression after 24 h of treatment ($p < 0.05$; Fig. 4B). As a consequence, the *OPG*/*RANKL* ratio, which is a predictor of osteoclastogenesis, was significantly elevated after 24 h of treatment, with the highest level at 72 h ($p < 0.05$; Fig. 4C).

Mineralization assay

The hPDL cells were grown in osteogenic induction medium, with or without HU-308, for a period of 21 d. Alizarin Red staining revealed that the HU-308-treated group exhibited accelerated accumulation of mineralized nodules, whereas the control group showed less deposited mineralized nodules (Fig. 5).

Discussion

The CB2 signaling in the skeleton is one of the most important recent findings in the field of bone metabolism. It has been demonstrated that activation of CB2 contributes to the maintenance of bone mass by stimulation of bone formation and inhibition of bone resorption (17,18). In this study, we investigated CB2 expression and the effect of CB2 activation on osteogenic differentiation of hPDL cells for the first time.

The periodontium includes two kinds of fibroblasts: gingival fibroblasts

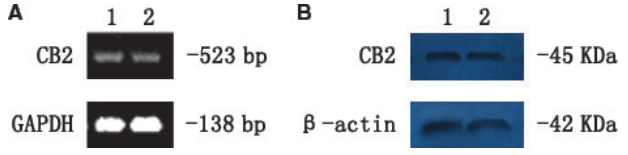

Fig. 1. Expression of CB2 in hPDL cells assessed by RT-PCR and Western blotting analysis. (A) In lane 1, CB2 mRNA expression was detected in hPDL cells using RT-PCR analysis. In lane 2, MC3T3 E1 cells were used as the positive control. (B) In lane 1, CB2 protein expression was observed in hPDL cells using Western blotting analysis. In lane 2, MC3T3 E1 cells were used as the positive control. The results are representative of three independent experiments.

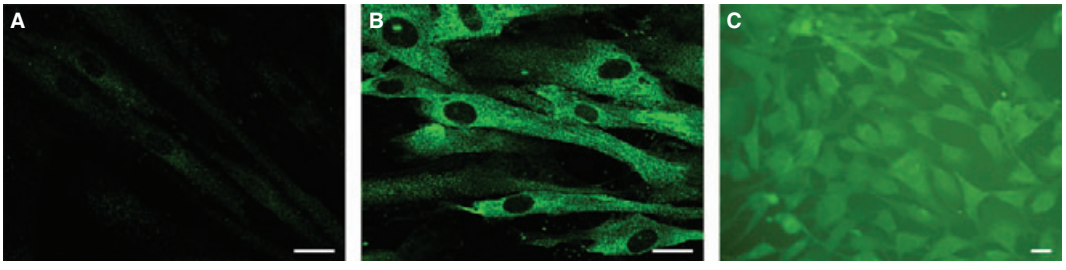

Fig. 2. Expression of CB2 in hPDL cells assessed by immunofluorescence assay. (A) Fairly mild green fluorescence was detected in the negative control, in which normal rabbit serum was used instead of primary antibody against CB2. (B) Clear green fluorescence of CB2 protein was observed in hPDL cells. (C) MC3T3 E1 cells were used as the positive control. Scale bars represent 20 μm.

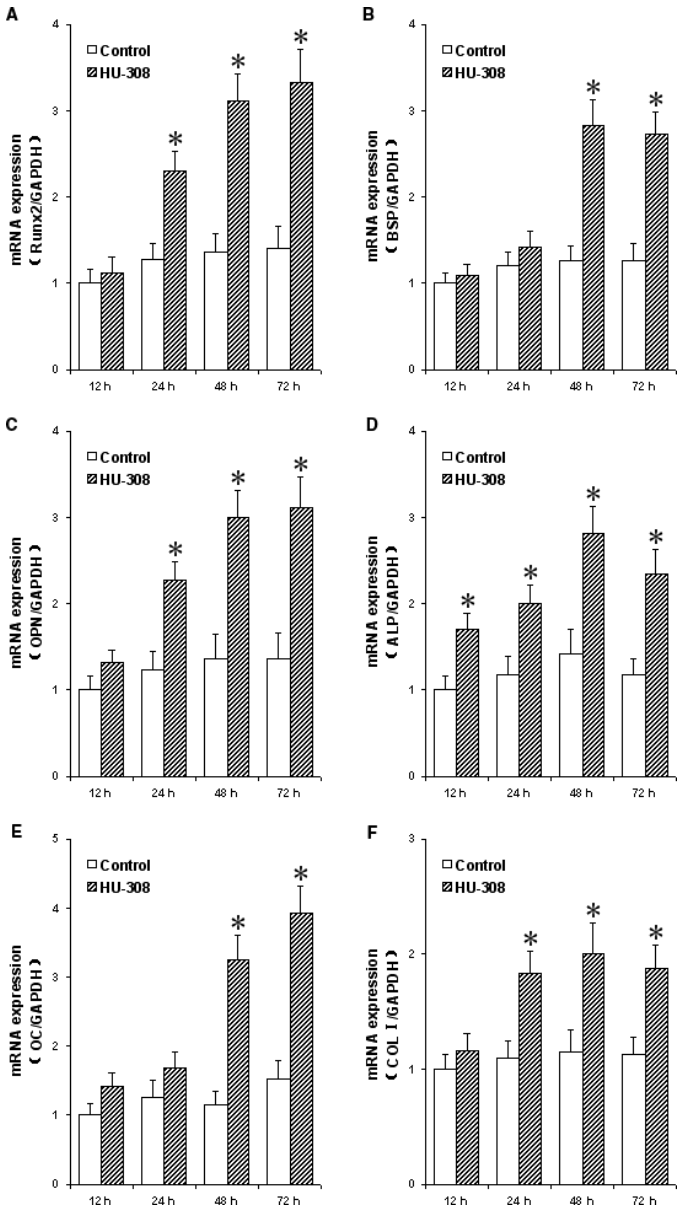

Fig. 3. Effect of HU-308 on the mRNA expression of osteogenic genes in hPDL cells. The mRNA of *Runx2* (A), *BSP* (B), *OPN* (C), *ALP* (D), *OC* (E) and *COL I* (F) in hPDL cells was subjected to quantitative real-time PCR analysis following HU-308 (10^{-7} M) treatment for 12, 24, 48 or 72 h. The data are presented as means ± SD from three independent experiments. *$p < 0.05$ vs. the control group.

and PDL cells (25). Although the two cell types may exhibit distinct phenotypic characteristics (5,21), they both establish a dynamic balance between tissue formation and degradation at the tooth–bone interface. Nakajima *et al.* (22) have reported that human gingival fibroblasts express CB2 and that activation of CB2 has an anti-inflammatory effect on human gingival fibroblasts. In the present study, we discovered that hPDL cells also expressed CB2. To date, there have been no reports on CB2 expression in hPDL cells. Our study has therefore provided the first evidence that hPDL cells express CB2. Since CB2 plays a crucial role in the regulation of bone metabolism as well as inflammation (17,18,22), we further explored the effect of CB2 activation on osteogenic differentiation of hPDL cells.

Since the discovery of the endocannabinoid system, several endocannabinoids, such as anandamide (26), 2-arachidonoylglycerol (13,27), 2-arachidonyl glyceryl ether (noladin ether; 28) and virodhamine (29), have been identified to bind to CB2 in physiological conditions. Nakajima *et al.* (22) have discovered that in the healthy state human gingival crevicular fluid contains a low level of anandamide, and the anandamide concentration is increased in pathological conditions. They pointed out that anandamide may exude from the blood and/or arise through local release from periodontal tissue as a result of infection by gram-negative microorganisms (22). In our study, a CB2-specific agonist, HU-308, was used to activate CB2 in hPDL cells. HU-308 is a synthetic, highly specific cannabinoid ligand for CB2 (30). It has been reported that activation of CB2 by HU-308 stimulates osteogenic activity of osteoblasts and restrains osteoclast formation (17). Owing to the certified effect of HU-308 on osteogenesis and osteoclastogenesis, we selected it to investigate the effect of CB2 activation on osteogenic and osteoclastogenic gene expression in hPDL cells.

In the present study, we found that activation of CB2 by HU-308 promoted expression of osteogenic genes, including *Runx2*, *BSP*, *OPN*, *ALP*, *OC* and *COL I*, in hPDL cells, consequently leading to accelerated mineralization. Ofek *et al.* (17) have indicated that HU-308 accelerates osteogenic activity of osteoblasts by increased tissue non-specific ALP activity and mineralized nodule formation. The process of osteogenic differentiation consists of three phases: proliferation with matrix formation (1–7 d); maturation (7–12 d); and mineralization (12–21 d; 31). During this process, orchestrated expression of osteogenic genes takes place. ALP, as an early marker for osteogenic differentiation, is a marker enzyme for differentiation of osteogenic cells (32,33). Runx2 is an osteoblast-specific transcription factor, which is implicated as a major regulator of osteoblast differentiation and gene expression (34). OC is a vitamin K-dependent Ca^{2+}-binding protein of

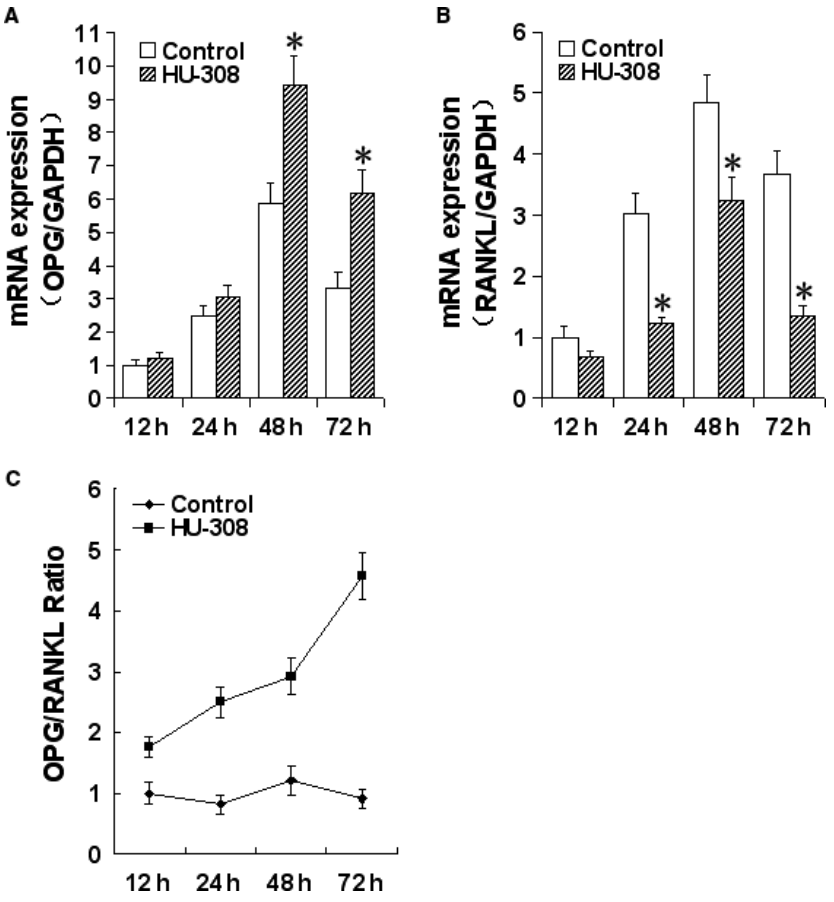

Fig. 4. Effect of HU-308 on the mRNA expression of osteoclastogenic genes in hPDL cells. The mRNA of *OPG* (A) and *RANKL* (B) in hPDL cells was subjected to quantitative real-time PCR analysis following HU-308 (10^{-7} M) treatment for 12, 24, 48 or 72 h. (C) Ratio of *OPG* to *RANKL*. The data are presented as means ± SD from three independent experiments. *$p < 0.05$ vs. the control group.

bone matrix and OPN functions as the modulator of matrix mineralization, whereas COL I and BSP have been shown to serve as a mineralization scaffold and a hydroxyapatite initiation site, respectively (35–37). Therefore, our results show that activation of CB2 can enhance osteogenic differentiation of hPDL cells by increased expression of osteogenic genes and accumulation of mineralized matrix.

Osteoprotegerin and RANKL, both expressed in osteoblasts, have been shown to play a key role in the regulation of osteoclastogenesis. RANKL binds its receptor, RANK, on the osteoclast precursor surface, determining its activation and differentiation into a mature osteoclast (38–41). OPG exerts its effect by acting as a RANKL decoy receptor, thus preventing its binding to osteoclasts and inhibiting osteoclast activation (38,42). It is accepted that *OPG* and *RANKL* are two essential osteoclastogenic genes in the regulation of osteoclastogenesis, and the ratio of *OPG*/*RANKL* determines osteoclast differentiation and activation (43,44). The hPDL cells express osteoprotegerin (6) and RANKL (7), suggesting that they may regulate OPG through the OPG/RANKL system. Low ratios were observed in hPDL cells from resorbing deciduous teeth (45) and in periodontal tissue from patients with advanced periodontitis (46). Ofek *et al.* (17) have found that activation of CB2 by HU-308 restrains osteoclastogenesis both directly, by suppression of osteoclast formation, and indirectly, by inhibition of osteoblast RANKL expression. Recently, Napimoga *et al.* (47) have reported that cannabidiol, a cannabinoid component from cannabis sativa, decreases bone resorption by inhibiting

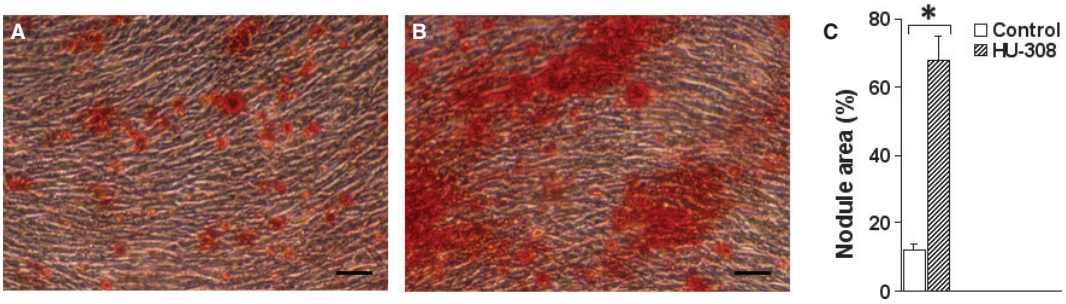

Fig. 5. Mineralization assay of hPDL cells grown in osteogenic induction medium, with or without HU-308 (10^{-7} M), for a total of 21 d. (A) The hPDL cells in osteogenic induction medium without HU-308 showed less deposited mineralized nodules as measured by Alizarin Red staining. (B) The hPDL cells grown in osteogenic induction medium with HU-308 exhibited accelerated accumulation of mineralized nodules. (C) The nodule area was significantly higher in the HU-308-treated group, compared with that in the control group. The data are presented as means ± SD from three independent experiments. *$p < 0.05$ vs. the control group. Scale bars represent 100 μm.

RANK/RANKL expression of alveolar bone during experimental periodontitis in rats. In this study, we observed that HU-308 up-regulated OPG expression and down-regulated RANKL expression in hPDL cells, contributing to the elevated OPG/RANKL ratio. Since the ratio of OPG to RANKL can be indicative for the role of hPDL cells in bone resorption or prevention of bone resorption (45,46), our results suggest that activation of CB2 in hPDL cells could inhibit osteoclastic activity, potentially creating a favorable osteogenic microenvironment.

In conclusion, our findings demonstrate that activation of CB2 is able to enhance osteogenic differentiation of hPDL cells and potentially create a favorable osteogenic microenvironment. This implies that CB2 might play an important role in alveolar bone metabolism. Our study provides a potential rationale for the use of exogenous factors, such as CB2-specific agonists, as bone promoters in the remodeling and regeneration of alveolar bone. Further studies are required to corroborate the effect of CB2 activation on alveolar bone metabolism *in vivo*.

Acknowledgements

This study was supported by National Natural Science Foundation of China (Grant No. 30600610).

References

1. Williams RC. Periodontal disease. *N Engl J Med* 1990;**322:**373–382.
2. Somerman MJ, Young MF, Foster RA, Moehring M, Imm GR, Sauk JJ. Characteristics of human periodontal ligament cells *in vitro*. *Arch Oral Biol* 1990;**35:**241–247.
3. Arceo N, Sauk JJ, Moehring J, Foster RA, Somerman MJ. Human periodontal cells initiate mineral-like nodules *in vitro*. *J Periodontol* 1991;**62:**499–503.
4. Nojima N, Kobayashi M, Sionome M, Takahashi N, Suda T, Hasegawa K. Fibroblastic cells derived from bovine periodontal ligament have the phenotypes of osteoblasts. *J Periodontal Res* 1990; **25:**179–185.
5. Ivanovski S, Li H, Haase HR, Bartold PM. Expression of bone associated macromolecules by gingival and periodontal ligament fibroblasts. *J Periodontal Res* 2001;**36:**131–141.
6. Wada N, Maeda H, Tanabe K *et al*. Periodontal ligament cells secrete the factor that inhibits osteoclastic differentiation and function: the factor is osteoprotegerin/osteoclastogenesis inhibitory factor. *J Periodontal Res* 2001;**36:**56–63.
7. Kanzaki H, Chiba M, Shimizu Y, Mitani H. Dual regulation of osteoclast differentiation by periodontal ligament cells through RANKL stimulation and OPG inhibition. *J Dent Res* 2001;**80:**887–891.
8. Kawarizadeh A, Bourauel C, Götz W, Jäger A. Early responses of periodontal ligament cells to mechanical stimulus *in vivo*. *J Dent Res* 2005;**84:**902–906.
9. Wescott DC, Pinkerton MN, Gaffey BJ, Beggs KT, Milne TJ, Meikle MC. Osteogenic gene expression by human periodontal ligament cells under cyclic tension. *J Dent Res* 2007;**86:**1212–1216.
10. Lekic PC, Rajshankar D, Chen H, Tenenbaum H, McCulloch CA. Transplantation of labeled periodontal ligament cells promotes regeneration of alveolar bone. *Anat Rec* 2001;**262:**193–202.
11. Seo BM, Miura M, Gronthos S *et al*. Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet* 2004;**364:**149–155.
12. Squitieri F, Cannella M, Sgarbi G *et al*. Severe ultrastructural mitochondrial changes in lymphoblasts homozygous for Huntington disease mutation. *Mech Ageing Dev* 2006;**127:**217–220.
13. Mechoulam R, Ben-Shabat S, Hanus L *et al*. Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem Pharmacol* 1995;**50:**83–90.
14. Howlett AC. The cannabinoid receptors. *Prostaglandins Other Lipid Mediat* 2002; **68–69:**619–631.
15. Tam J, Trembovler V, Di Marzo V *et al*. The cannabinoid CB1 receptor regulates bone formation by modulating adrenergic signaling. *FASEB J* 2007;**22:**285–294.
16. Bab I. The skeleton: stone bones and stoned heads? In: Mechoulam R, ed. *Cannabinoids as Therapeutics. Milestones in Drug Therapy Series*. Birkhäuser: Basel, 2005:201–206.
17. Ofek O, Karsak M, Leclerc N *et al*. Peripheral cannabinoid receptor, CB2, regulates bone mass. *Proc Natl Acad Sci U S A* 2006;**103:**696–701.
18. Scutt A, Williamson EM. Cannabinoids stimulate fibroblastic colony formation by bone marrow cells indirectly via CB2 receptors. *Calcif Tissue Int* 2007;**80:**50–59.
19. Karsak M, Cohen-Solal M, Freudenberg J *et al*. Cannabinoid receptor type 2 gene is associated with human osteoporosis. *Hum Mol Genet* 2005;**14:**3389–3396.
20. Yamada Y, Ando F, Shimokata H. Association of candidate gene polymorphisms with bone mineral density in community-dwelling Japanese women and men. *Int J Mol Med* 2007;**19:** 791–801.
21. Somerman MJ, Archer SY, Imm GR, Foster RA. A comparative study of human periodontal ligament cells and gingival fibroblasts *in vitro*. *J Dent Res* 1988; **67:**66–70.
22. Nakajima Y, Furuichi Y, Biswas KK *et al*. Endocannabinoid, anandamide in gingival tissue regulates the periodontal inflammation through NF-κB pathway inhibition. *FEBS Lett* 2006;**580:**613–619.
23. Inanc B, Elcin AE, Elcin YM. Osteogenic Induction of human periodontal ligament fibroblasts under two- and three-dimensional culture conditions. *Tissue Eng* 2006;**12:**257–266.
24. Miura M, Gronthos S, Zhao M *et al*. SHED: stem cells from human exfoliated deciduous teeth. *Proc Natl Acad Sci USA* 2003;**100:**5807–5812.
25. Giannopoulou C, Cimasoni G. Functional characteristics of gingival and periodontal ligament fibroblasts. *J Dent Res* 1996; **75:**895–902.
26. Devane WA, Hanus L, Breuer A *et al*. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science* 1992;**258:**1946–1949.
27. Sugiura T, Kondo S, Sukagawa A *et al*. 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. *Biochem Biophys Res Commun* 1995; **215:**89–97.
28. Hanus L, Abu-Lafi S, Fride E *et al*. 2-arachidonyl glyceryl ether, an endogenous agonist of the cannabinoid CB1 receptor. *Proc Natl Acad Sci USA* 2001;**98:**3662–3665.
29. Porter AC, Sauer JM, Knierman MD *et al*. Characterization of a novel endocannabinoid, virodhamine, with antagonist activity at the CB1 receptor. *J Pharmacol Exp Ther* 2002;**301:**1020–1024.
30. Hanus L, Breuer A, Tchilibon S *et al*. HU-308: a specific agonist for CB (2), a peripheral cannabinoid receptor. *Proc Natl Acad Sci U S A* 1999;**96:**14228–14233.
31. Afzal F, Polak J, Buttery L. Endothelial nitric oxide synthase in the control of osteoblastic mineralizing activity and bone integrity. *J Pathol* 2004;**202:**503–510.
32. Wlodarski KH, Reddi AH. Alkaline phosphatase as a marker of osteoinductive cells. *Calcif Tissue Int* 1986;**39:**382–385.
33. Beertsen W, van den Bos T. Calcification of dentinal collagen by cultured rabbit periosteum: the role of alkaline phosphatase. *Matrix* 1989;**9:**159–171.

34. Yamaguchi A, Komori T, Suda T. Regulation of osteoblast differentiation mediated by bone morphogenetic proteins, hedgehogs, and Cbfa1. *Endocr Rev* 2000; **21:**393–411.
35. Beresford JN, Gallagher JA, Poser JW, Russell RG. Production of osteocalcin by human bone cells *in vitro*. Effects of 1,25(OH)2D3,24,25(OH)2D3, parathyroid hormone, and glucocorticoids. *Metab Bone Dis Relat Res* 1984;**5:**229–234.
36. Franceschi RT, Iyer BS. Relationship between collagen synthesis and expression of the osteoblast phenotype in MC3T3-E1 cells. *J Bone Miner Res* 1992;**7:**235–246.
37. de Oliveira PT, Zalzal SF, Irie K, Nanci A. Early expression of bone matrix proteins in osteogenic cell cultures. *J Histochem Cytochem* 2003;**51:**633–641.
38. Simonet WS, Lacey DL, Dunstan CR *et al*. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell* 1997;**89:**309–319.
39. Yasuda H, Shima N, Nakagawa N *et al*. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc Natl Acad Sci USA* 1998;**95:**3597–3602.
40. Tsuda E, Goto M, Mochizuki S *et al*. Isolation of a novel cytokine from human fibroblasts that specifically inhibits osteoclastogenesis. *Biochem Biophys Res Commun* 1997;**234:**137–142.
41. Lacey DL, Timms E, Tan HL *et al*. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* 1998;**93:**165–176.
42. Yasuda H, Shima N, Nakagawa N *et al*. Identity of osteoclastogenesis inhibitory factor (OCIF) and osteoprotegerin (OPG): a mechanism by which OPG/OCIF inhibits osteoclastogenesis *in vitro*. *Endocrinology* 1998;**139:**1329–1337.
43. Hofbauer LC, Khosla S, Dunstan CR, Lacey DL, Boyle WJ, Riggs BL. The roles of osteoprotegerin and osteoprotegerin ligand in the paracrine regulation of bone resorption. *J Bone Miner Res* 2000;**15:**2–12.
44. Crotti T, Smith MD, Hirsch R *et al*. Receptor activator NF kappaB ligand (RANKL) and osteoprotegerin (OPG) protein expression in periodontitis. *J Periodontal Res* 2003;**38:**380–387.
45. Fukushima H, Kajiya H, Takada K, Okamoto F, Okabe K. Expression and role of RANKL in periodontal ligament cells during physiological root-resorption in human deciduous teeth. *Eur J Oral Sci* 2003;**11:**346–352.
46. Liu D, Xu JK, Figliomeni L *et al*. Expression of RANKL and OPG mRNA in periodontal disease: possible involvement in bone destruction. *Int J Mol Med* 2003;**11:**17–21.
47. Napimoga MH, Benatti BB, Lima FO *et al*. Cannabidiol decreases bone resorption by inhibiting RANK/RANKL expression and pro-inflammatory cytokines during experimental periodontitis in rats. *Int Immunopharmacol* 2009;**9:**216–222.