จุฬาลงกรณ์มหาวิทยาลัย

ทุนวิจัย
กองทุนรัชดาภิเษกสมโภช

รายงานผลการวิจัย

IL-1β กระตุ้นปฏิสัมพันธ์ระหว่างเซลล์เคลือบรากฟันกับต้นกำเนิดของเซลล์ละลายกระดูก
(สัญญาเลขที่ R/F_๒๕๕๗_๐๒๑_๐๓_๓๒)

โดย

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กิตติกรรมประกาศ

Special thanks to Prof. Prasit Pavasant and Prof. Vincent Evert for their kind support.

บทคัดย่อภาษาอังกฤษ

Project Title IL-1β mediate cementoblasts and osteoclast precursors interaction

Name of the Investigator Assoc.Prof. Ruchanee Ampornaramveth

Year April 2016

An injury of the periodontium followed by an inflammatory response often leads to root resorption. Resorption is accomplished by osteoclasts and their generation may depend on an interaction with the cells in direct contact with the root, the cementoblasts. Our study aimed to investigate the role of human cementoblasts in the formation of osteoclasts and the effect of IL-1β hereupon. Extracted teeth from healthy volunteers were subjected to sequential digestion by type I collagenase and trypsin. The effect of enzymatic digestion on the presence of cells on the root surface was analyzed by histology. Gene expression of primary human cementoblasts (pHCB) was compared with a human cementoblast cell line (HCEM). The pHCBs were analyzed for their expression of IL-1 receptors as well as of RANKL and OPG. In a co-culture system consisting of osteoclast precursors (blood monocytes) and pHCBs, the formation of osteoclasts and their resorptive activity was assessed by osteo-assay and ivory slices. The cells obtained after a 120 min enzyme digestion expressed the highest level of bone sialoprotein; similar to that of HCEM. This fraction of isolated cells also shared a similar expression pattern of IL1 receptors (IL-R1 and IL-R2). Treatment with IL-1β potently upregulated RANKL expression but not of OPG. pHCBs were shown to induce the formation of functional

osteoclasts. This capacity was significantly stimulated by pre-treating the pHCBs with IL-1β prior to their co-culture with human blood monocytes. Our study demonstrated that cementoblasts have the capacity to induce osteoclastogenesis; a capacity strongly promoted by IL-1β. These results may explain why osteoclasts can be formed next to the root of teeth.

บทคัดย่อภาษาไทย

ชื่อโครงการวิจัย IL-1β กระตุ้นปฏิสัมพันธ์ระหว่างเซลล์เคลือบรากฟันกับต้นกำเนิดของเซลล์ละลายกระดูก

ชื่อผู้วิจัย รศ.ทญ.ดร. รัชนี อัมพรอร่ามเวทย์

เดือนและปีที่ทำวิจัยเสร็จ เมษายน 2559

การบาดเจ็บของอวัยวะปริทันต์มักจะตามมาด้วยการอักเสบซึ่งส่งผลให้เกิดการละลายของรากฟัน การละลายนี้เกิดจากการก่อตัวของเซลล์ละลายกระดูก ซึ่งเป็นกระบวนการที่จะเกิดขึ้นได้เมื่อมีปฏิสัมพันธ์ระหว่างเซลล์ที่อยู่ติดกับรากฟัน ซึ่งก็คือเซลล์เคลือบรากฟัน การศึกษานี้มุ่งที่จะหาความสัมพันธ์และบทบาทของเซลล์เคลือบรากฟันในการกระตุ้นการเจริญและพัฒนาของเซลล์ละลายกระดูกและผลของอินเตอร์ลิวคินวันเบต้า ฟันที่ถูกถอนจากอาสาสมัครที่มีสุขภาพแข็งแรงจะถูกนำไปย่อยด้วยเอนไซม์คอลลาจิเนสชนิดที่ 1 และทริปซิน ผลของการย่อยด้วยเอนไซม์ต่อการปลอดปล่อยเซลล์ถูกตรวจสอบด้วยวิทยาเนื้อเยื่อ การแสดงออกของยีนของเซลล์เคลือบรากฟันที่แยกได้ถูกเปรียบเทียบกับเซลล์ไลน์ของเซลล์เคลือบรากฟัน การแสดงออกของยีน IL-1 receptors 1 และ 2 รวมทั้ง RANKL และ OPG ในเซลล์เคลือบรากฟันถูกวิเคราห์ การเจริญพัฒนาไปเป็นเซลล์ละลายกระดูกของเซลล์ต้นกำเนิดเซลล์ละลายกระดูกในเลือดถูกตรวจสอบในสภาพวะที่ถูกเลี้ยงร่วมกับเซลล์เคลือบรากฟัน และความสามารถในการละลายกระดูกถูกตรวจสอบ เซลล์ที่แยกได้จากการย่อยด้วยเอนไซม์ที่ 120 นาทีมีการแสดงออกของยีน bone sialoprotein ในระดับเดียวกับเซลล์ไลน์ของเซลล์ละลายกระดูก และเซลล์กลุ่มนี้มีการแสดงออกของ IL-1R1 และ IL-1R2 ที่ใกล้เคียงกัน IL-1β สามารถกระกระตุ้นการแสดงออกของ RANKL แต่ไม่มีผลต่อ OPG เซลล์เคลือบรากฟันที่แยกได้สามารถกระตุ้นการเจริญและพัฒนาของเซลล์ละลายกระดูกได้ และความสามารถนี้ถูกกระตุ้นด้วยได้ด้วยการใส่ IL-1β ลงไปในอาหารเลี้ยงเซลล์ล่วงหน้า 24 ชั่วโมงก่อนนำไปเลี้ยงร่วมกับเซลล์ต้นกำเนิดของเซลล์ละลายกระดูกจากเลือด การศึกษานี้แสดงให้เห็นว่าเซลล์เคลือบรากฟันมีความสามารถในการกระตุ้นการเจริญและพัฒนาของเซลล์ละลายกระดูกได้

และความสามารถนี้ถูกส่งเสริมด้วย IL-1β ผลการศึกษานี้อาจช่วยอธิบายว่าทำไมเซลล์ละลายกระดูกจึงสามารถเกิดขึ้นได้รอบๆรากฟัน

สารบัญ

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Table 1: Primers used in RT-PCR.

Primer	Sequence ID	Sequence (Forward and Reverse 5'-3')	Base pairs	Cycles
ALP	NM_000478.4	F: CGA GAT ACA AGC ACT CCC ACT TC R: CTG TTC AGC TCG TAC TGC ATG TC	121	35
BSP	NM_004967.3	F: GAT GAA GAC TCT GAG GCT GAG A R: TTG ACG CCC GTG TAT TCG TA	514	38
DMP-1	NM_004407.3	F: CAG GAG CAC AGG AAA AGG AG R: CTG GTG GTA TCT TGG GCA CT	213	38
GAPDH	NM 002046.3	F: TGA AGG TCG GAG TCA ACG GAT R: TCA CAC CCA TGA CGA ACA TGG	396	22
IL-1β	NM 000576.2	F: GGA GCA ACA AGT GGT GTT CT R: AAA GTC CAG GCT ATA GCC GT	458	32
IL1-R1	NM 000877.2	F: AGG AGA CGG AGG ACT TGT GT R: GCG TCA TAG GTC TTT CCA TC	755	30
IL1-R2	NM 173343.1	F: TCC TGC CGT TCA TCT CAT ACC R: TCC ATG TGC AAA TCC TCT CTT	573	40
OPG	NM 002546.3	F: TCA AGC AGG AGT GCA ATC G R: AGA ATG CCT CCT CAC ACA GG	341	24
RANKL	NM 003839.2	F: GCC AGC TAG AAA ACC ACC AA G: TGG ATT TGC TTC CAG GCT CA	517	40

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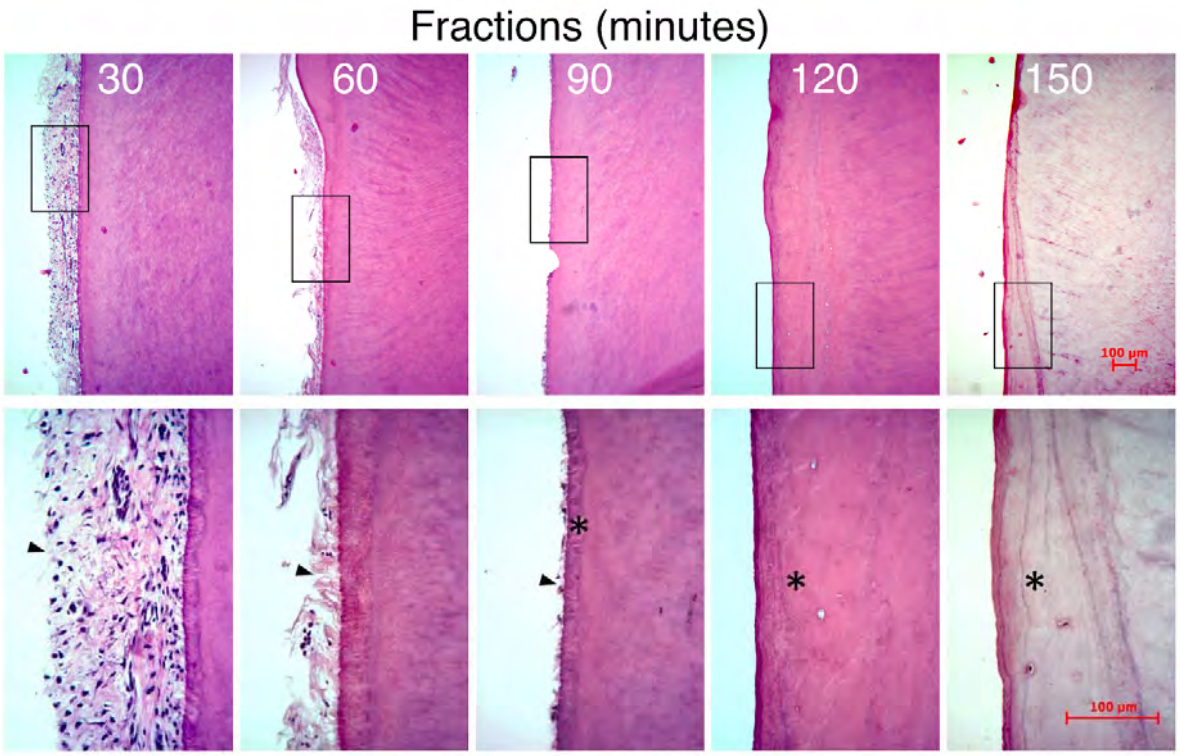

Fig. 1. Histologic section, H&E staining of the root surface after enzymatic digestion at each time point, 30-min, 60-min, 90-min, 120-min and 150-min (arrowhead: cells, asterisk: cementum, scale bar 100 μm).

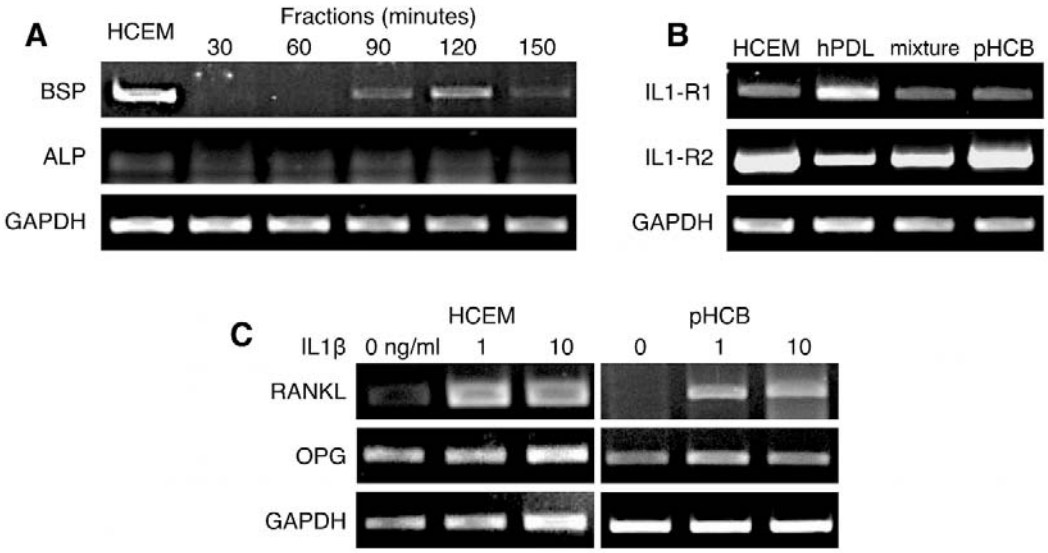

Fig. 2. mRNA expression of cells collected from different fractions of enzymatic digestion of tooth. A) BSP, DMP-1 and ALP expression. B) IL1 receptor 1 and receptor 2 expression. C) RANKL and OPG expression after treatment with IL1β (HCEM: human cementoblast cell line, hPDL: primary human periodontal ligament cells, pHCB: primary human cementoblasts; mixture: cells from 60-90min fractions).

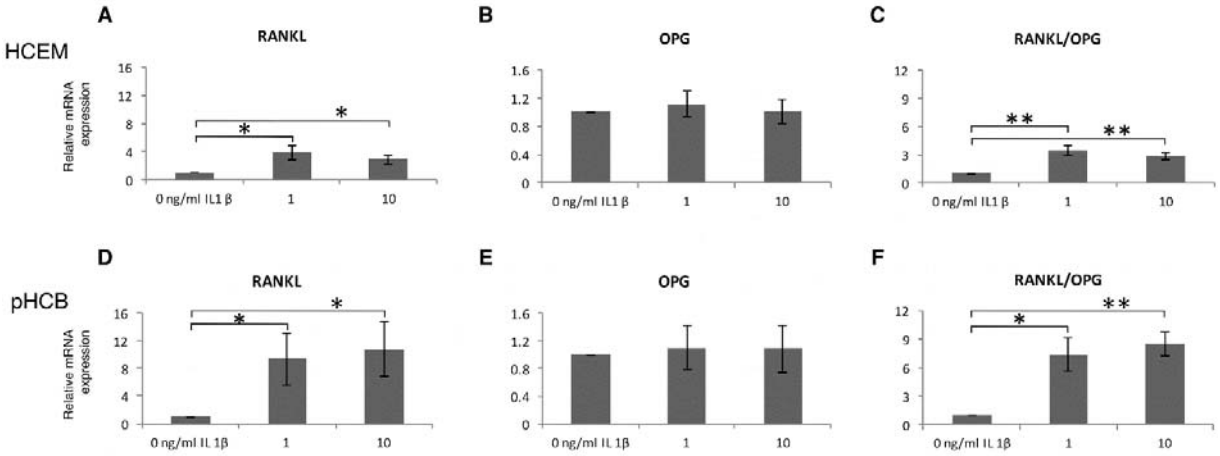

Fig. 3. The effect of IL1β on osteoclast associated genes. A) RANKL, B) OPG and C) RANKL/OPG ratio of HCEM. D) RANKL, E) OPG and F) RANKL/OPG ratio of pHCB by semi quantitive RT-PCR (one-way ANOVA, Dunnett's T3 post-hoc test, * $p < 0.05$; ** $p < 0.01$, n=4).

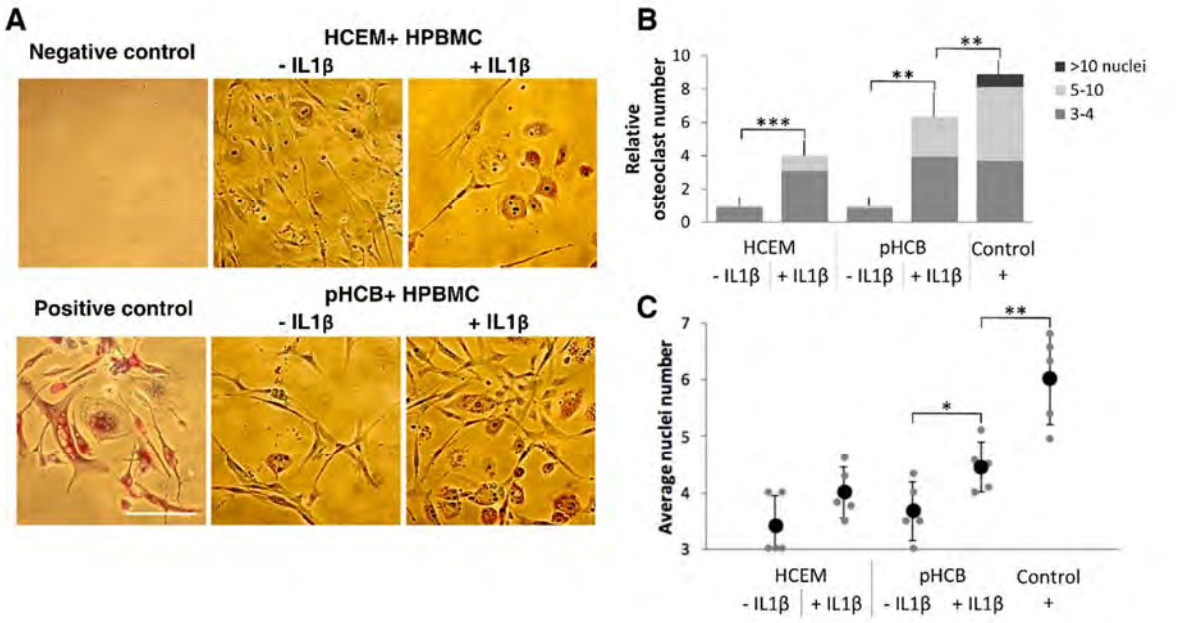

Fig. 4. Co-culture of cementoblasts and primary human peripheral blood monocytes for 21 days. A) TRAP and nuclear staining (scale bar 100 μm). B) Osteoclast number and the number of cells including different nuclei number. C) The average number (black dots) of nuclei per generated osteoclasts (independent samples comparison t-test, n=5).

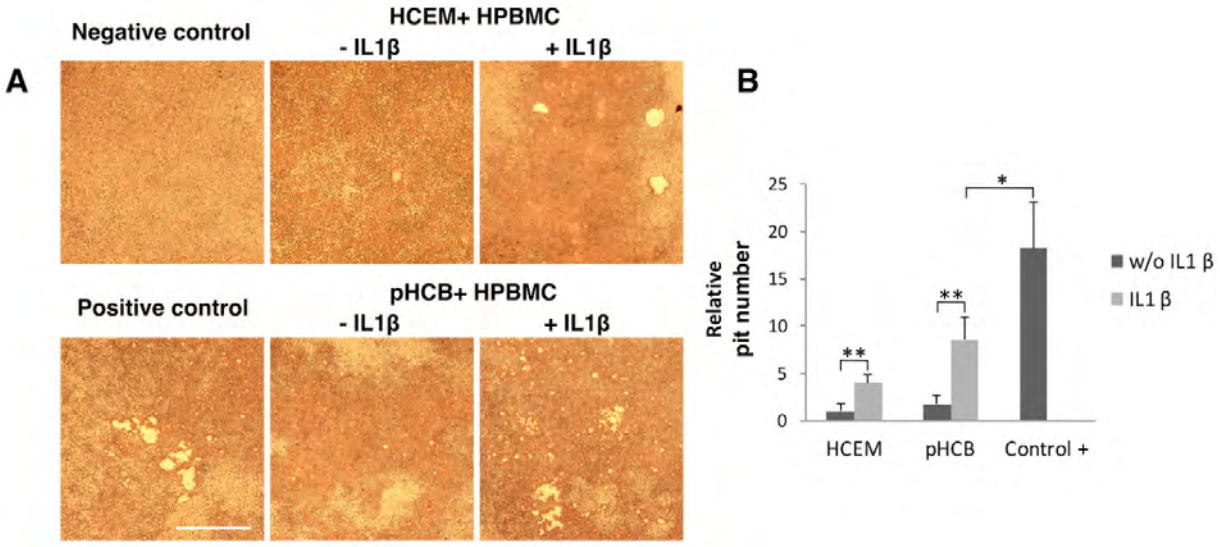

Fig. 5. Pit resorption assay by Osteo-Assay Plate: co-culture of cementoblasts and primary human peripheral blood monocytes for 21 days. A) Pit resorption (scale bar 100 μm). B) Fold change of pit resorption number (independent samples comparison t-test, n=4).

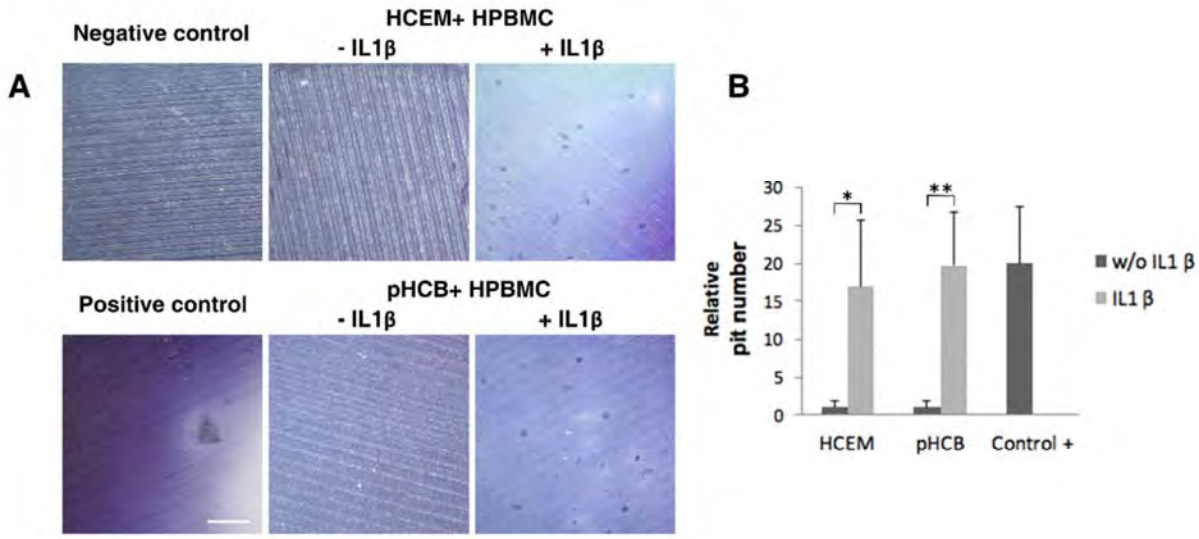

Fig. 6. Pit resorption assay by ivory slices: co-culture of cementoblasts and primary human peripheral blood monocytes for 21 days. A) Pit resorption stained with 1% toluidine blue in 1% sodium borate (scale bar 200 μm). B) Fold change of pit resorption number (independent samples comparison t-test, n=4).

Content

Introduction

Bone and cementum share a couple of similar characteristics such as structure and composition and also the cells responsible for their formation, osteoblasts and cementoblasts, are quite alike. In fact, cementoblasts, like osteoblasts, express genes for bone-regulatory molecules such as type I collagen, bone sialoprotein, osteopontin, dentin matrix protein 1, osteocalcin, and bone gla protein (Bosshardt, 2005). Yet, there is at least one unique difference between the two mineralized tissues, bone is remodeled whereas cementum is not. Under physiologic conditions, both cellular and acellular cementum increase slowly in thickness, but the tissue is not digested like bone. In contrast to bone, the root surface does not harbor specialized cells capable of resorbing the cementum. However, as an effect of tissue injury induced by trauma, root resorption can occur.

Injury to periapical tissue can cause damage to periodontal cells (cementoblasts and/or PDL cells) after which a local formation is induced of cementoclasts/osteoclasts (Schjott and Andreasen, 2005). During this process of injury inflammatory cytokines are likely to be released at these sites. IL-1β is one of the most prominent inflammatory cytokines and it has been reported to play

an important role in inflammatory bone loss of the periodontium. Its expression was elevated in gingival crevicular fluid at sites of recent bone and attachment loss in patients with periodontal disease (Graves, 2008). An incubation of periodontal ligament (PDL) fibroblasts with IL-1β resulted in an upregulation of RANKL expression (Bloemen et al., 2011). RANKL is essential for the formation of osteoclasts and several studies have shown that these cells are indeed generated in the presence of PDL fibroblasts (Fukushima et al., 2005; Shimizu et al., 2000). It is not clear, however, whether also cementoblasts have the capacity to induce the formation of osteoclasts. Although osteoclast formation was shown to occur in a co-culture of osteoclast precursors and a mouse cementoblast cell line (Oka et al., 2007), no data are available on such a role played by primary human cementoblasts. In the present study we investigated this by isolating cementoblasts form human third molars and their co-culture with mononuclear blood cells. Osteoclast formation as well as their resorptive activity was analyzed both in the absence and presence of IL-1ß.

Procedure

Isolation and culturing of primary human cementoblasts

Third molars from healthy young individuals, age 18 to 25-year-old, were extracted as recommended by their dentists. Each subject was without systemic and oral infection or diseases and the molars had no caries. The patients provided written consent for the use of discarded tissue for research purposes. Tissue samples were de-identified and analyzed anonymously. The Ethics Committee of the Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand approved the study to be carried out according to the protocol and informed permission dated and/or amended as follows in compliance with the ICH/GCP (HREC-DCU 2014-002).

Immediately after extraction, each tooth was transferred to ice cold storage medium (10% FBS, 1% L-Gluamine, 0.5 mg/ml gentamicin and 3 mg/ml amphotericin B in DMEM, #11960, Gibco, Life Technologies Corporation, Grand Island, NY) and transported to the lab. To avoid contamination, soft tissue attached to the cervical area was carefully removed. Then the extracted molars were rinsed twice in Dulbecco's phosphate-buffered saline without calcium and magnesium (PBS).

In order to obtain cells from the root surface, the tooth was immersed in a digestion solution containing type I collagenase (2 mg/ml, #17100-017, Gibco), and 0.25% trypsin in serum free

medium (1% BSA in DMEM) and incubated at 37°C. The released cells were collected every 30 minutes and the digestion solution was changed. The sequential digestion continued up to 150 minutes, thus included five consecutive digestion steps. The cells from each fraction were centrifuged and immersed in growth medium (10% FBS, 1% antibiotics in αMEM, #12000-022, Gibco). The cells were cultured at 37°C humidified atmosphere with 5% CO2, the medium was replaced every 3 days. The primary human cementoblasts (pHCB) at the 3rd–6th passages were used for the following experiments. hPDL cells were cultured from the first fraction of digestion (30 minutes). Human cementoblast cell line (HCEM) was a gift from Professor Takashi Takata (Hiroshima University). The cell line was cultured under the same conditions as used with the primary cells.

Demineralized tooth section preparation

To examine the presence of cells that remained on the root surface following sequential enzymatic digestion, demineralized tooth sections of each time points were made and stained with hematoxylin and eosin (H&E). The teeth were fixed in 10% formaldehyde neutral buffer solution overnight, decalcified in a 10% EDTA solution (pH 7.4) for 2 weeks at 4°C, embedded in paraffin and cut at 5 micrometer thickness.

IL-16 incubation

pHCBs and HCEM were seeded in 24-well plates. After 80% confluence, 1 or 10 ng/ml recombinant human IL-1β (#201-LB-005, R&D Systems, Minneapolis, MN) was added and the cells were subsequently cultured for 24 hr at 37°C with 5% CO_2.

RNA isolation and semi-quantitative reverse transcription–polymerase chain reaction (RT-PCR)

To assess the expression of marker genes, semi-quantitative RT-PCR was performed as previously described (Huynh et al., 2016; Osathanon et al., 2013). Total mRNA was extracted using Trizol reagent. The cells were scraped off the plate and collected. The lysate was extracted by adding 100 µl chloroform, mixed and centrifuged for 15 minutes at 14,000 rpm. mRNA was precipitated with 250 µl isopropanol and the pellet was dissolved in nuclease free water (DEPC). The amount of RNA was measured using a spectrophotometer (NanoDrop2000, Thermo Scientific, Wilmington, DE).

First-strand cDNA was synthesized using reverse transcriptase reaction by ImProm-II Reverse Transcription System (#A3800, Promega Corporation, Madison, WI). Semi-quantitative PCR was performed using Tag polymerase (Tag DNA Polymerase, Invitrogen, Thermo Fisher Scientific) with a reaction volume of 25 µl containing 25 pmol of primers and 1 µl of RT product. The amplification profile was one cycle at 94°C for 1 min, hybridization

at 60°C for 1 min and extension at 72°C, followed by 1 extension cycle of 10 min at 72°C. The PCR was performed in the DNA thermal cycler (Biometra UNO-Thermoblock Thermal Cycler PCR DNA, Biometra GmbH, Göttingen, Germany) and subsequently the amplification cycle was repeated 22 –40 times. The cycle number was determined so that the PCR product levels were within a linear range. The amplified DNA was then electrophoresed on a 1.8% agarose gel and visualized by ethidium bromide fluorostaining (#1610433, Bio-Rad Laboratories, Hercules, CA).

PCR primers for alkaline phosphatase (ALP), bone sialoprotein (BSP), and dentin matrix acidic phosphoprotein 1 (DMP-1) were used as markers of cementoblasts, interleukin 1 (IL1-β) receptor type I and II (IL1-R1, IL1-R2) were used to screen endogenous expression in HCEM, pHCB and human periodontal ligament cells (hPDL cells). Receptor activator of nuclear factor kappa-B ligand (RANKL) and osteoprotegerin (OPG) gene expression were used to evaluate the effect of IL-1β incubation (Table 1). All bands were scanned, analyzed, and normalized with the expression of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using Bio-1D software version 15.03 (Vilber Lourmat, Marne La Vallée, France). Three independent experiments were repeated in each sample.

Harvest of human peripheral blood monocytes and co-culture with cementoblasts

Human peripheral blood monocytes (hPBMCs) were isolated according to a previously described protocol (Bloemen et al., 2011). Peripheral venous blood was drawn from a healthy male donor, using heparin as anticoagulant (10 IU/ml) (#9041-081, Sigma-Aldrich). The blood was diluted 1:1 with RPMI 1640 (#8070, Gibco). Twenty-five ml diluted blood cell suspension was gently pipetted on top of 12.5 ml of Histopaque (#1077, Sigma-Aldrich) followed by centrifugation at 900 g for 30 minutes at room temperature. The white blood cell ring fraction was transferred to a new 50 ml tube using a sterile plastic pipette, the volume was adjusted to 50 ml using PBS and centrifuged at 1200 rpm for 10 minutes. The supernatant was discarded and the pellet was resuspended using RPMI Medium 1640 before counting the cells.

For co-culture experiments, human cementoblasts were seeded at $2.6x10^3$ cells/cm^2 in 24-well plates overnight. On the next day, the cells were incubated with 1 ng/ml IL-1β for 24 hr. On the following day, hPBMCs were seeded at $5x10^4$ cells/cm^2 onto the IL-1β stimulated cementoblasts. Control cultures were cultured without the cytokine. The cultures were kept in αMEM, 10% heat inactivated FBS (#16000-036, Gibco), 10^{-8} M 1,25-dihydroxyvitamin D3 (#D1530, Sigma-Aldrich), 10^{-7} M

dexamethasone and 25ng/ml recombinant human M-CSF (#574802, BioLegend, San Diego, CA). The co-cultures were maintained at 37°C with 5% CO_2 for 21 days. A positive control group consisted of a monoculture of HPBMCs in the same medium plus 25 ng/ml recombinant human RANKL (#591102, BioLegend). A negative control group consisted of a monoculture of HPBMCs in αMEM supplemented with 10% FBS.

Tartrate-resistant acid phosphatase (TRAcP) staining for osteoclasts

After 21 days of culture, the cells were fixed with 10% formalin, neutral buffer for 5 minutes at room temperature and stained for TRAcP activity as well as their nuclei (#MK300, TAKARA BIO INC., Shiga, Japan). Briefly, the cells were washed 3 times with distilled water, stained with chromogenic substrate (NABP/FRVLB), sodium tartrate buffer and incubated at 37°C for 45 minutes followed by nuclear staining for 5 minutes. Finally, cells were washed with distilled water and observed for multinucleated TRAcP-positive cells (brightly red stained). Micrographs were taken from five different areas per well randomly. The number of TRAcP-positive multinucleated cells (≥ 3 nuclei) was assessed.

Bone resorption assay in vitro

The functionality of the osteoclasts was analyzed by using two different resorption assays: Osteo-assay Plates (#3987, Corning,

NY) and ivory slices. The Osteo-assay plates are coated with inorganic 3-dimensional crystalline material (bone biomimetic synthetic surface). Ivory slices were cut with a low speed water-cooled diamond saw. The slices had a diameter of 7 mm and and a thickness of 0.5 mm. Osteoclasts were generated from hPBMCs in mono- and co-cultures with cementoblasts as described above. After 21 days of culture, the cells were lysed by 2.5% chlorinated soda solution for 5 minutes at room temperature and washed by distilled water. Ivory slices were stained with a solution of 1% toluidine blue (#89640, Sigma-Aldrich) in 1% sodium borate (#S9640, Sigma-Aldrich) for 4 min. Resorption pits were observed and quantified using photomicrographs from five randomly chosen fields by inverted phase contrast microscopy for Osteo-assay plates and stereomicroscopy Discovery V8 (Zeiss, Oberkochen, Germany) for the ivory slices.

Data analysis

Each experiment was repeated at least three times. We calculated the means and standard deviations (SD) for each set of data. For statistical analyses, independent samples comparison t-test, one-way ANOVA, and Dunnett's T3 post-hoc tests were used to compare between groups using SPSS v.21 (IBM) with the level of significance at $P < 0.05$

Result

Isolation and characterization of primary human cementoblasts

Sequential enzymatic digestion was performed to isolate pHCBs. The released cells were collected every 30 minutes for five consecutive digestions. In order to evaluate how enzymatic digestion affected the layer of cells on the root surface, sections of the teeth were examined by H&E staining. The thickness of ligament and cellular layer on the root surface gradually decreased with the increasing digestion time. At 90 min of digestion only a single layer of cells remained associated with cementum (Fig. 1). After 150 min incubation no cells were detected anymore, indicating that the cells present at the 120 min time interval were the cementoblasts. These cells were isolated during the 120 min incubation.

To characterize the cells from each fraction they were compared with the human cementoblast cell line (HCEM), and the expression of BSP, DMP-1 and ALP was evaluated. BSP was not expressed in the early fractions (30- and 60-min). Low levels of expression were seen in the 90-min fraction and a peak was found in the 120-min fraction (Fig. 2a). The expression of BSP of the latter cells was comparable to that of the HCEM. The expression of DMP-1 and ALP was comparable for all samples. The cementoblast

phenotype corresponded to their BSP expression profile resembling that of the HCEM. We therefore considered the cells from 120-min fraction as primary human cementoblasts and used these for our further experiments.

IL-1β treatment induced RANKL expression and increased RANKL/OPG ratio in human cementoblasts

In order to investigate the response of cementoblasts to IL-1β, we examined first whether these cells expressed IL-1β and its receptors. pHCB, HCEM, hPDL and the cells from the 60 and 90 min isolations (these contain both hPDL and pHCB) did not express IL-1β (data not shown) but expressed both types of the IL1-receptor, type I and II. pHCB and HCEM expressed IL1-R1 and IL1-R2 comparably whereby a stronger expression of IL1-R2 was observed. This pattern was distinct from hPDLs; these cells expressed comparable levels of IL1-R1 and IL1-R2 (Fig. 2b).

IL-1β increased RANKL mRNA expression in both pHCB and HCEM. There was no difference in OPG expression upon IL-1β treatment (Fig. 2c). These responses resulted in an increased ratio of RANKL/OPG (Fig. 3).

IL-1β stimulated cementoblast induced osteoclastogenesis

To determine whether cementoblasts had the capacity to induce osteoclastogenesis and the effect of IL-1β hereupon, they were co-cultured with osteoclast precursors, hPBMCs. Cementoblasts were or were not pre-cultured with IL-1β for 24 hrs prior to their co-culture with hPBMCs. The co-cultures were kept for 21 days in media containing M-CSF, Vit D3 and dexamethasone. Mono-cultures of hPBMCs were cultured in medium that contained RANKL, M-CSF, Vit D3 and dexamethasone. These latter cultures were used as positive control.

In the absence of IL1-β, the number of TRAcP positive multinucleated cells was low. The number of cells with 3-4 nuclei was approximately 60 cells/cm^2. A 24 hr pre-treatment of the cementoblasts with IL-1β significantly increased the number of TRAcP positive multinucleated cells (Fig. 4a-4b). The number of TRAcP positive cells with 3-4 and 5-10 nuclei was 275 and 170 cells/cm^2, respectively. The osteoclasts generated in the co-culture groups were smaller than those generated in the presence of exogenous RANKL, the positive control group. The diameter of the osteoclasts generated in the presence of the cementoblasts was around 50 μm, whereas those generated in the presence of exogenous RANKL had a diameter of around 100 μm.

It was of interest to note that the osteoclasts generated in the co-culture with cementoblasts were found in close association with

the latter cells (Fig. 4a). The majority of osteoclasts generated in the co-cultures contained 3-4 nuclei, only a low percentage of cells had 5-10 nuclei. In the cultures of PBMCs to which RANKL was added (positive control group) the majority of cells contained 5-10 nuclei or even more than 10 nuclei per cell. In summary, a pre-treatment of pHCBs with IL-1β stimulated the formation of multinucleated osteoclasts. Yet, the addition of exogenous RANKL resulted in a higher number of TRAcP positive cells with more nuclei per cell (Fig. 4c).

IL-1β pre-treated cementoblasts induced functional resorbing osteoclasts

To analyze an important functional aspect of the multinucleated cells formed, resorption of a mineralized surface, two different types of pit assays were performed. The cementoblasts were cultured as indicated above, but they were seeded on Osteo-assay plates or on ivory slices. Resorption was found in co-cultures with or without a pre-incubation with IL-1β (Fig. 5-6). Yet, the co-cultures in which the cementoblasts were pre-incubated with the cytokine, showed a significantly higher level of resorption (Fig.5-6). The positive control group (monoculture of hPBMCs in medium supplemented with RANKL) showed the highest level of resorption. Analysis of the ivory slices revealed the presence of resorption pits

(Fig. 6). The number of these pits was significantly higher in the IL-1β pre-incubated cementoblasts culture and comparable to the number in the positive control group (Fig.6).

Discussion

Our study, for the first time, demonstrated the capacity of primary human cementoblasts to induce the formation of functional osteoclasts. We found that cementoblasts did indeed induce multinucleated TRAcP positive cells that resorbed a mineralized substratum. Thus these cells were actual osteoclasts. An essential question is whether the cells isolated from the tooth root surface were cementoblasts. By using a sequential enzymatic digestion, the cementum-lining cells were collected and characterized (Kaneda et al., 2006; MacNeil et al., 1998). In addition, histologic analysis was performed to investigate the cells that remained on the root surface after each incubation. The results clearly demonstrated that after 120-min of digestion no cells occupied the root surface. With some discrepancy of the specimens, we were able to obtain small number of cells from 150 min fraction for gene expression analysis. Cells collected in the 120-min fraction were considered as the last cellular layer lining the root surface, thus cementoblasts. The mRNA expression of BSP, a major protein component of cementum, confirmed the cementoblast phenotype. This expression was similar to that of an immortalized cell line from a previous study (Kitagawa et al.,

2006). The cells from the outer layer, primarily PDL cells, did not express BSP whereas the cells from 90-min fraction expressed a low level. These findings are in line with data presented previously on the expression of BSP by cementum-associated cells (Gao et al., 1999; Kaneda et al., 2006). The latter authors showed that BSP is primarily expressed by cells lining the root surface, the cementoblasts, but not by PDL cells. Our finding might indicate that cementoblasts resemble more osteoblasts than PDL cells (Huynh et al., 2016; Kaneda et al., 2006). Moreover, our pHCB expressed a similar pattern of DMP-1 and ALP mRNA as HCEM and PDL cells. Expression of DMP-1 has been shown for a cementoblast cell line and undifferentiated primary PDL cells (Huynh et al., 2016; Nociti et al., 2014; Wang et al., 2015). Meanwhile, several studies showed that cementoblasts expressed a similar level of ALP mRNA as PDL cells, but expressed a higher ALP activity (Groeneveld et al., 1995; Kaneda et al., 2006; Li et al., 2001).

To investigate whether the formation of osteoclasts as induced by cementoblasts was modulated by the pro-inflammatory cytokine, IL1-β, we first investigated whether human cementoblasts expressed IL-1β and its receptors. Apparently the cementoblasts

and PDL cells did not express IL-1β. This finding is in line with other studies where only a weak expression of the cytokine by mesenchymal cells was noted (Sumanasinghe et al., 2009), whereas high levels are produced by activated macrophages and various immune cells (Sumanasinghe et al., 2009). Both IL-1ß receptors were expressed at a comparable level by the cementoblasts and the cell line while the expression by hPDL cells differed. IL1-R1 generally mediates most of the responses to IL1 whereas IL1-R2 has been reported to function as a decoy receptor (Graves and Cochran, 2003). Our study demonstrated a difference in expression of IL-R1 and IL-R2 in cementoblasts and PDL cells. This finding might suggest that these two cell types respond differently to IL-1. PDL cells can stimulate osteoclastogenesis by expression of RANKL and inhibit this process by OPG. In this way these fibroblasts can affect processes such as periodontitis and orthodontic tooth movement (Kanzaki et al., 2001). The difference in expression of IL1-R1 and IL1-R2 might imply distinctive properties of cementoblasts and PDL cells. The higher expression of R2 receptor in cementoblasts might in-directly demonstrate its protective role to the root surface. This cell type might be less sensitive to the inflammatory

cytokine. Furthers studies are needed to explore this possibility in more detail.

In several studies using a mouse cementoblast cell line (OCCM-30) an upregulation of RANKL/OPG was shown to occur under the influence of different compounds such as sclerostin and PGE2. These findings suggested that this cell type can indeed induce osteoclastogenesis (Bao et al., 2013; Mada et al., 2006; Oka et al., 2007). Bao et al. (2013) demonstrated the inhibition of sclerostin on OPG expression in this mouse cementoblast cell line (Bao et al., 2013). Oka et al. (2007) showed that PGE2 promoted cementoblast-mediated cementoclastogenesis by regulating the expression of RANKL and OPG via the EP4 pathway (Oka et al., 2007). In contrast to our findings, Kim et al. (2008) reported that human cementoblasts do not have the capacity to induce osteoclastogenesis. These authors co-cultured, however, the cells with mouse bone marrow cells (Kim et al., 2008). A possible explanation for the lack of osteoclast formation is the source of osteoclast precursors used. It is not unlikely to assume that the interaction between human cementoblasts and mouse cells is quite different from an interaction of such cementoblasts with human blood-derived precursors, as used in our study.

Although macrophages and osteoclasts have been detected in the periodontal space, still little is known about the origin of newly recruited osteoclasts (Xie et al., 2008). Resorption requires specific interactions between various cell types and hard tissue. Multinucleated osteoclasts are formed as a result of injury to bone, cementum, or dentin (Yamaguchi, 2009). Prior to the onset of osteoclastogenesis, osteoclast precursors are stimulated by macrophage colony-stimulating factor (M-CSF) to undergo mitosis while RANKL/OPG modulates their differentiation into multinucleated osteoclasts (Gartner et al., 2011; Schoppet et al., 2002). We successfully demonstrated that human-derived cementoblasts, even without the presence of an inflammatory cytokine, were able to induce osteoclast formation. This ability was enhanced if the cells were exposed to the inflammatory cytokine, IL-1β. The effect was seen already at fairly low concentrations of the cytokine. Previously it was shown that stimulation of PDL fibroblasts with 1, 10, 100 ng/ml IL-1β had a long-lasting effect, leading to a significantly increased osteoclastogenesis after a short and single exposure of these cells to the cytokine (Bloemen et al., 2011). Thus both types of

periodontal cells, fibroblasts and cementoblasts, respond to the cytokine by inducing relatively high numbers of osteoclasts.

In our co-culture model we found fewer and smaller osteoclasts when compared to a mono-culture of osteoclast precursors with a continuous supply of recombinant RANKL. The number of nuclei per cell was also lower of osteoclasts generated in the co-cultures. This may represent osteoclasts with a somewhat lower resorptive activity. The different in outcome is more likely due to the expression of RANKL induced by a single dose exposure to IL-1β is lower than the amount of added RANKL. Situation might exacerbate in some pathologic condition that induce continuous supply of IL-1β in surrounding tissue (Graves, 2008). It is questionable whether this approach mimics an actual pathological condition that occur *in vivo.* Under these conditions the cells are probably continuously exposed to the inflammatory cytokine. In previous studies, induction of osteoclasts by a co-culture with PDL cells, the average number of nuclei per osteoclast was higher than in our study suggesting that PDL cells might have a stronger osteoclastogenic supporting ability compared to cementoblasts (de Vries et al., 2006). The higher number in PDL co-cultures is in accordance with the stronger up-regulation of RANKL/OPG in

response to pro-inflammatory cytokines by PDL cells (de Vries et al., 2006; Fukushima et al., 2005).

The multinucleated cells generated in the co-cultures proved to be functional osteoclasts. These cells not only resorbed the mineralized layer in the osteo-assay but also generated resorption pits in ivory slices. The formation of the pits is a strong evidence for the nature of these cells being indeed osteoclasts. The demineralization of the osteo-assay plates could have been caused by macrophages (Jeganathan et al., 2014; Tumber et al., 2003), but the actual degradation of the ivory can only be performed by osteoclasts. The results showed further that IL-1β stimulated the resorption, an effect noted previously (Bloemen et al., 2011). Other reports showed IL-1β-dependent induction of RANKL and BSP in human cementoblasts under compressive force mimicking orthodontic tooth movement (Diercke et al., 2012a; Diercke et al., 2012b). While Nemeto et al. suggested that murine cementoblasts participate in the recruitment of osteoclastic precursor cells via TLR-2 under LPS treatment in the inflammatory response (Nemoto et al., 2006). Further study need to investigate mechanism the effect of IL-1β on

osteoclastogenesis of human cementoblasts as well as compare with osteoblast during the root tooth resorption.

Direct cell-cell interaction between osteoclast precursors and PDL fibroblasts significantly modulates the cellular response which favors the expression of osteoclast-related gene expression and the ultimate formation of osteoclasts (Bloemen et al., 2010; Bloemen et al., 2011). In fact, the presence of cementoblast-like or fibroblast-like stromal cells in association with odonto/osteoclasts has been reported in root resorption of deciduous teeth (Sasaki, 2003). It is, however, not very likely that under physiological conditions cementoblasts and precursors of osteoclasts have a frequent chance to come into direct contact. Under pathologic conditions such a situation is more likely to occur.

Conclusion

Our study demonstrated the capacity of human cementoblasts to induce osteoclastogenesis; an effect strongly promoted by the pro-inflammatory cytokine IL-1β. The study provides a clue to explain root resorption in traumatized teeth.

Suggestion for Further Work

Role of cementoblast to induce osteoclastic activity under pathologic root/bone resorption should be conduct by utilizing in vivo model.

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