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Expression of functional Toll-like receptors and nucleotide-binding oligomerization domain proteins in murine cementoblasts and their upregulation during cell differentiation

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Background and Objective: While the primary role of cementoblasts is to synthesize the components of cementum, we have reported that immortalized murine cementoblasts (OCCM-30) express functional Toll-like receptor (TLR)-2 and -4, and these receptors are involved in the alteration of gene expression associated with cementum formation and in the upregulation of osteoclastogenesis-associated molecules, such as receptor activator of nuclear factor-κB (NF-κB) ligand. We hypothesized that cementoblasts express a wide range of pattern recognition receptors in a manner comparable to osteoblasts, which are known to express various functional TLRs and nucleotide-binding oligomerization domain (NOD) proteins.

Material and Methods: Murine cementoblasts and pre-osteoblasts were used. The gene and protein levels of TLRs/NODs were analyzed using real-time polymerase chain reaction and flow cytometry. Interleukin-6 (IL-6) and activated NF-κB were measured using enzyme-linked immunosorbent assay.

Results: The expressions of TLR-1, -2, -4, -6 and -9, CD14, NOD-1 and -2 were detected in cementoblasts and were upregulated upon differentiation induced by ascorbic acid. Similar patterns were observed in the mouse MC3T3-E1 osteoblast cell line. Synthetic ligands, Pam3CSK4 (TLR-1/2 agonist), Pam2CGDPKHPKSF (TLR-2/6 agonist), lipid A (TLR4 agonist), CpG DNA (TLR-9 agonist), FK565 (NOD1 agonist) and muramyldipeptide (NOD2 agonist), effectively induced NF-κB activation in cementoblasts and/or ascorbic acid-treated cementoblasts. Furthermore, these ligands induced IL-6 production in a NF-κB-dependent manner in cementoblasts and/or ascorbic acid-treated cementoblasts.

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Conclusion: These results indicate that cementoblasts possess functional TLR and NOD signaling systems and have a similar capacity to osteoblasts in responding to a wide variety of pathogens.

The innate immune system is initiated by the recognition of conserved motifs in pathogens termed 'pathogen-associated molecular patterns' (PAMPs). Members of the Toll-like receptor (TLR) family and CD14 are essential components in this process. To date, at least 12 members of the TLR family have been identified in mammalian systems as pattern recognition receptors (PRRs; 1). Toll-like receptor-4 recognizes lipopolysaccharide (LPS) from gram-negative bacteria (2). Toll-like receptor-2 recognizes a diverse set of pathogen-associated motifs including peptidoglycan, lipoproteins/lipopeptides and lipoteichoic acid from various microorganisms (1). It has been demonstrated that this broader ligand specificity (3) is attributed to the unique ability of TLR-2 to heterodimerize with TLR-1 and TLR-6 for the recognition of triacylated and diacylated lipoprotein, respectively (4,5). Toll-like receptor-9 recognizes bacterial DNA in a manner dependent on unmethylated cytosin-phosphorothioate-guanine (CpG) dinucleotides in particular base contexts, which are termed CpG motifs (6). CD14 not only facilitates the binding of LPS to the TLR-4–myeloid differentiation protein (MD)-2 complex (7), but has also been reported to bind directly to triacylated lipopeptides and facilitate recognition of the lipopeptides by the TLR-2–TLR-1 complex (8). Recently, a family of nucleotide-binding oligomerization domain (NOD) proteins has been identified as intracellular PRRs. NOD1 interacts with motifs found in peptidoglycans carrying *meso*-diaminopimelic acid (*meso*-DAP), which is found in most gram-negative and some gram-positive bacteria (9,10). In contrast, NOD2 has been implicated as a general sensor of bacterial peptidoglycans because it recognizes a minimal motif present in almost all peptidoglycans, muramyldipeptides MurNAc-L-Ala-D-isoGln (MDP) or MurNAc-L-Ala-D-Glu (11,12).

Cementum is a thin mineralized tissue covering the tooth root surface that shares many properties with bone (13) and most notably a remarkable similarity in biochemical composition (14). However, cementum differs from bone in its histology by lacking innervation and vascularization. In addition, cementum has no bone marrow and limited remodeling potential when compared with bone, where a system consisting of osteoblasts, osteocytes, bone-lining cells and osteoclasts is required (15).

While the primary role of cementoblasts and osteoblasts is to synthesize the components of cementum and bone matrix, respectively, it has been reported that osteoblasts exhibit functional expression of TLR-2, TLR-4 (16,17), TLR-5 (18), TLR-9 (6), NOD1 (19) and NOD2 (19,20) and initiate the release of inflammatory cytokines and osteoclastogenesis by activating these receptors. For example, *Escherichia coli* LPS interacts with TLR-4 on the surface of osteoblasts and upregulates the expression of receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL) and downregulates the expression of osteoprotegerin (OPG), which acts as a decoy receptor for RANKL (17,21). The CpG oligodeoxynucleotides interact with osteoblastic TLR-9 and increase the expression of molecules regulating osteoclastogenesis (6). Furthermore, NOD2-mediated signals are involved in MDP-induced RANKL expression in osteoblasts (20). Recently, we reported that murine cementoblasts express functional TLR-2 and TLR-4 (22,23), and they were found to be involved in the alteration of gene expression associated with cementum formation (22) and the upregulation of osteoclastogenesis-associated molecules such as RANKL (23). These findings suggest that cementoblasts participate in the innate immune system in a way comparable to osteoblasts by expressing not only TLR-2 and TLR-4 but also other functional PRRs.

In the present study, we demonstrate that murine cementoblasts express TLR-1, -2, -4, -6 and -9, CD14, NOD1 and NOD2 and their adaptor molecules at the gene and/or protein levels and that the expression levels of PRRs are upregulated in the course of cell differentiation. Furthermore, we show that these PRRs are functional receptors using synthetic chemical ligands specific for the respective TLRs and NODs.

Material and methods

Reagents

Biotin-conjugated monoclonal antibodies (mAbs) for mouse TLR-1 (eBioTR23), mouse TLR-2 (T2.5, mouse IgG_1), mouse TLR-4/MD-2 (MTS510, Rat IgG_{2a}), fluorescein isothiocyanate (FITC)-conjugated mAbs for mouse TLR-9 (M9.D6), mouse CD14 (Sa2-8), rat IgG_{2a} isotype control antibody and phycoerythrin (PE)-conjugated streptavidin were purchased from eBioscience (San Diego, CA, USA). Anti-mouse TLR-6 (rabbit polyclonal) was from IMGENEX (San Diego, CA, USA). Anti-rabbit IgG (H + L; goat polyclonal, fluor 488) was from AnaSpec, Inc. (San Jose, CA, USA). Pyrrolidine dithiocarbamat (PDTC, a NF-κB inhibitor) was purchased from Calbiochem-Novabiochem Co. (La Jolla, CA, USA). All tissue culture reagents were purchased from Gibco BRL (Rockville, MD, USA).

Stimulants

Pam_3CSK_4 (TLR-1/2 agonist), a synthetic tripalmitoylated lipopeptide that mimics the acylated aminoterminus of bacterial lipoproteins, and Pam_2 CGDPKHPKSF (TLR-2/6 agonist), a synthetic lipoprotein representing the N-terminal part of the 44 kDa lipoprotein LP44 of *Mycoplasma salivarium*, were purchased from InvivoGen (San Diego, CA, USA). Synthetic muramyldipeptide [MurNAc-L-Ala-D-isoGln (MDP); NOD2 agonist] and *E. coli*-type lipid A (LA-

15-PP; TLR-4 agonist) were purchased from Protein Research Foundation Peptide Institute (Osaka, Japan). FK565 (heptanoyl-γ-D-Glu-*meso*-DAP-D-Ala; NOD1 agonist) was supplied by Astellas Pharmaceutical Co. (Tokyo, Japan). Conventional CpG DNA (TLR-9 agonist), CpG ODN 1826 [TCCATGACGTTCCTGACGTT (CpG motif is underlined)], was purchased from Sigma-Aldrich Japan K.K. (Tokyo, Japan).

Cells

An immortalized murine cementoblast cell line (OCCM-30), established by the isolation of tooth root-surface cells from transgenic mice containing an SV40 large T-antigen under the control of the osteocalcin (OCN) promoter (24), was kindly provided by Dr Martha J. Somerman, University of Washington, WA, USA. The OCCM-30 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Rockville, MD, USA) plus 10% fetal bovine serum (FBS; Gibco BRL) and antibiotics. A murine pre-osteoblastic cell line, MC3T3-E1, was purchased from ATCC (Manassas, VA, USA) and maintained in α-minimal essential medium (α-MEM; Gibco) containing 10% FBS and antibiotics. A murine macrophage cell line, J774-1, was obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan) and maintained in RPMI 1640 (Nissui pharmaceutical Co., Tokyo, Japan) containing 10% FBS and antibiotics. The experimental procedures were approved by the Ethical Review Board of Tohoku University Graduate School of Dentistry (Sendai, Japan).

Cell differentiation and treatment

To induce cell differentiation, confluent OCCM-30 or MC3T3-E1 cells were cultured in DMEM and α-MEM, respectively, containing 5% FBS in the presence of 50 μg/mL ascorbic acid (Sigma, St Louis, MO, USA) and the media were changed every 3 days. After reaching confluency, the cells were treated with the indicated synthetic stimulant in medium containing 5% FBS. In some experiments, cell stimulation was performed in the presence of 1 μM cytochalasin D (Sigma), where all the samples were adjusted to contain 0.1% (v/v) DMSO in the media.

Fluorescence activated cell sorting (FACS) analysis

Confluent cells were collected using Cell Dissociation Solution® (Sigma), washed with phosphate-buffered saline three times, and used for staining. For cell surface staining, a total of 10^5 cells were stained with each mAb or isotype-matched control IgG at 4°C for 20 min. Following washing, PE-conjugated streptavidin was added at 4°C for 20 min. Intracellular staining was performed with IC Fixation/Permeabilization buffer® (eBioscience) according to the manufacturer's instructions. Fixed and permeabilized cells were stained with FITC-conjugated mAbs or isotype-matched IgG for 20 min. Staining was analyzed on a FACSCalibur cytometer® (Becton Dickinson, Mountain View, CA, USA). The arithmetic mean was used in the computation of the mean fluorescence intensity (MFI).

Quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted using TRIzol® reagent (Gibco) according to the manufacturer's instructions, and was DNase-treated (DNA-free™; Ambion Inc., Austin, TX, USA). The transcription of total RNA into cDNA was carried out with the use of a Transcriptor First Strand cDNA Synthesis Kit® (Roche Diagnostic Co., Indianapolis, IN, USA) according to the manufacturer's instructions. The primer sequences for each gene, TLR-1, TLR-2, TLR-4, TLR-6, TLR-9, CD14, NOD1, NOD2, alkaline phosphatase (ALP), OCN and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), are shown in Table 1. The amplification profile was 40 cycles at 95/60; 55/30; 72/30 [temperature (°C)/time (s)]. The PCR was performed in an iCycler (Bio-Rad Laboratories, Hercules, CA, USA) with iQ SYBR Green Supermix (Bio-Rad) with optimized levels of 3 mM $MgCl_2$ and 0.5 μM of each primer. After amplification, one cycle of linear temperature gradient from 55 to 95°C at a transition rate of 0.5°C/30 s was performed to assess the specificity of the PCR products. For each run, water was used as the negative control. Reaction products were quantified with GAPDH as the reference gene. Since J774-1 and OCCM-30 are regarded as representative cells expressing functional TLRs/NODs and ALP/OCN, respectively, cDNA samples from these cells were used as calibrators for TLRs/NODs and ALP/OCN expression, respectively. Therefore, the value on the *Y* axis of the figures 2 and 3 indicates the fold-increase vs. the gene expression level in J774-1 or OCCM-30 cells. For conventional PCR, the

Table 1. Primer sequence used for polymerase chain reaction amplifications

Gene	Primer sequences forward/reverse
OCN	TGAACAGACTCCGGCG/GATACCGTAGATGCGTTTG
ALP	GGGGACATGCAGTATGAGTT/GGCCTGGTAGTTGTTGTGAG
TLR-1	TCTCTTCGGCACGTTAGC/CGTAAGAAATAAGAGCAGCCC
TLR-2	CGAGTGGTGCAAGTACG/GGTAGGTCTTGGTGTTCATTATC
TLR-4	GCAGCAGGTGGAATTGTATC/TGTTCTTCCTCTGCTGTTTG
TLR-6	CCGGTGGAGTACCTCAAT/TCAGCAAACACCGAGTATAGC
TLR-9	CTGCCGCTGACTAATCTG/CTGAAATTGTGGCCTATACCC
CD14	TCTCAGTTACAACAGGCTGGATA/CACTGCTTGGGATGATGG
NOD-1	GGACAACTTGCTGGAGAAT/CTGCAGCACGTAGAGGAA
NOD-2	CTTCATTTGGCTCATCCGTAG/CTGGAGATGTTGCAGTACAAAG
MyD88	TGATGCGGAGCCAGATT/GAGGAGGCATGTGTGTACT
TIRAP	GCTGAAGATGGGAACCAC/CTGCTGACCTTCCCGAT
IRAK4	CCAAATCTGACATCTACAGCTT/CATCCGTGTAATCTTCAATCG
Rip2 kinase	GGAGGAACAATCATCTATATGCC/ATGATCTGCAAAGGATTGGT
GAPDH	ACCACAGTCCATGCCATCAC/TCCACCACCCTGTTGCTGTA

amplification profile was 30 cycles using the same cycle programme (temperature/time). Amplified samples were visualized on 2.0% agarose gels stained with ethidium bromide and photographed under UV light.

Preparation of whole-cell extracts

Whole-cell extracts were prepared from confluent cells using a Nuclear Extract Kit™ (Active Motif, Carlsbad, CA, USA). Confluent cells cultured in six-well plates were harvested with a cell scraper and lysed in 75 μL of Complete lysis buffer. Cell suspensions were incubated on ice for 10 min on a rocking platform followed by centrifugation at 14,000 *g* for 20 min, and then the supernatants were collected and stored at −80°C until use. The concentration of protein in the cell lysates was measured using a *DC* Protein Assay® (Bio-Rad).

Enzyme-linked immunosorbent assay (ELISA)

The supernatants from cell cultures were harvested by centrifugation. The amount of IL-6 in the supernatant was measured using a mouse ELISA kit (BioSource International Inc., Camarillo, CA, USA). The assay was performed according to the manufacturer's instructions. Results were normalized to protein level using a standard curve. Each sample was assayed in triplicate.

Nuclear factor-κB ELISA

Activated NF-κB was measured with a TransAM™ NF-κB p65 transcription factor assay kit according to the manufacturer's instructions (Active Motif, Carlsbad, CA, USA). Briefly, whole-cell extracts (10 μg of protein per well) were added to 96-well plates coated with oligonucleotide containing the NF-κB consensus site (5′-GGGAC-TTTCC-3′) and incubated for 1 h at room temperature. After washing, the wells were incubated with NF-κB antibody for 1 h at room temperature followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG for 1 h at room temperature. Colorimetric reactions were developed and measured at 450 nm. For a positive control, the provided Jurkat cell nuclear extract (2.5 μg of protein per well) was used. Results were expressed as means of the value of optical density at 450 nm of triplicate measurements.

Statistical analysis

All experiments in this study were performed three times to test the reproducibility of the results, and representative findings are shown. Experimental values are given as means ± SD. The significance of differences between control and treatments was evaluated by one-way ANOVA. Values of $p < 0.05$ were considered significant.

Results

Expression of TLRs and CD14 at the protein level in cementoblasts and osteoblasts

We examined the possible expression of PRRs in cementoblasts and osteoblasts by flow cytometry. Confluent cultured (designated as day 0 cells) cementoblasts as well as osteoblasts expressed TLR-2, TLR-9 and CD14. The levels of TLR-2 expression in these cells were substantially comparable with that in the murine macrophage cell line, J774-1, which was used as a positive control, but the levels of TLR-9 and CD14 were much lower than that in J774-1. When the cells were cultured with ascorbic acid for 5 days (designated as day 5 cells), the expression levels of TLR-2 and TLR-9 in cementoblasts and the expression of TLR-9 and CD14 in osteoblasts were enhanced. Unlike J774-1 cells, which expressed significant levels of TLR-1, TLR-4 and TLR-6, both types of day 0 cells showed limited or slight expression of TLR-1, TLR-4 and TLR-6. Levels of TLR-1 and TLR4 in both cell types and TLR-6 in osteoblasts increased upon differentiation (Fig. 1).

Expression of TLRs, CD14 and NODs at the gene level in cementoblasts and osteoblasts

We next examined the gene expression of PRRs, including NOD1 and NOD2, in the course of cell differentiation. Cementoblasts were induced to differentiate by culturing in the presence of ascorbic acid over a 5 day period with ALP and OCN used as reference index genes (Fig. 2A). During cell differentiation, all PRRs were upregulated, with

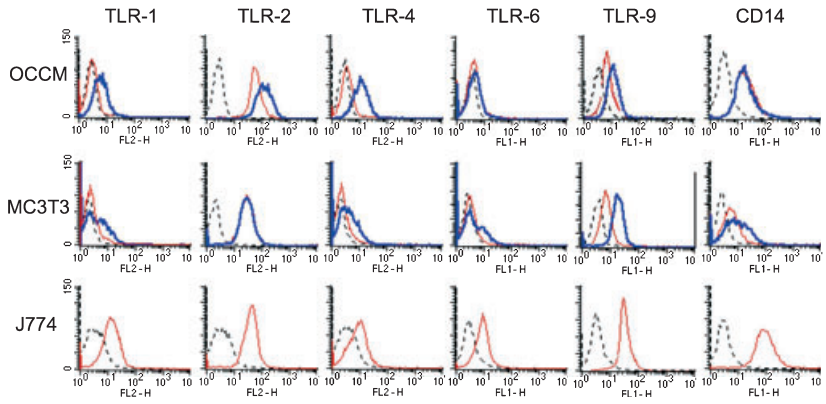

Fig. 1. Expression of TLR-1, -2, -4, -6 and -9 and CD14 at the protein level in cementoblasts, osteoblasts and macrophage cell lines. The OCCM-30 (cementoblasts) and MC3T3-E1 cells (osteoblasts) were cultured in DMEM and α-MEM, respectively, containing 5% FBS until confluent (day 0 cells; red lines), and then differentiation was induced by the addition of ascorbic acid (50 μg/mL) for 5 days (day 5 cells; blue line). The J774-1 cells (macrophage cell line) were cultured in RPMI 1640 containing 5% FBS until subconfluent (red line). After being harvested by cell dissociation solution (non-enzymatic), cells were stained with each TLR and CD14 antibody and analyzed by FACS. Representative data of three separate experiments are shown. Dotted line represents the isotype control staining (FL1:FITC, FL2:PE).

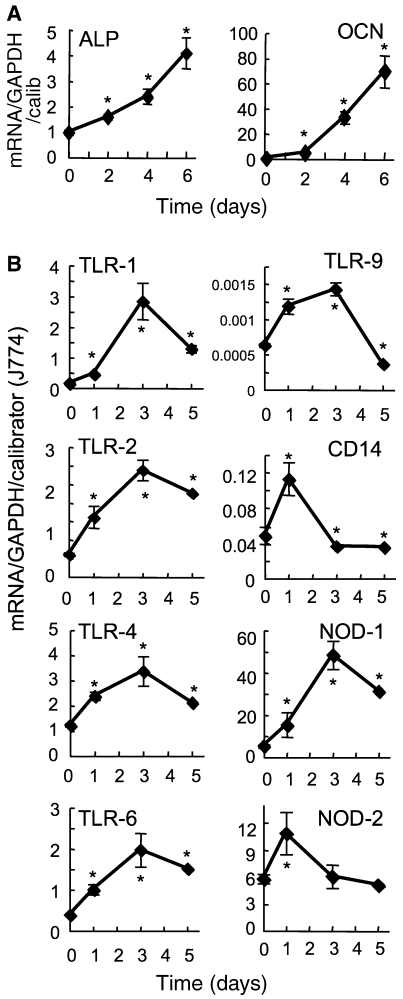

Fig. 2. Expression of TLR-1, -2, -4, -6 and -9, CD14, NOD1 and NOD2 at the gene level in cementoblasts. Confluent OCCM-30 cells were cultured in DMEM containing 5% FBS in the presence of ascorbic acid (50 μg/mL) for the indicated time periods. Total cellular RNA was extracted, and the mRNA expression levels were analyzed by real-time quantitative RT-PCR. Representative data of three separate experiments are shown as means ± SD of triplicate assays. Statistical significance is shown (*$p < 0.05$ vs. day 0 control).

a peak at days 1–3 (Fig. 2B). The expressions of TLR-9 and CD14 were lower by approximately three and one order(s) of magnitude, respectively, compared with those of J774-1, which is consistent with the protein expression levels shown in Fig. 1. The gene expression level of TLR-6, which was limited at the protein level in cementoblasts (days 0 and 5) shown in Fig. 1, was detected at day 0 and was significantly induced upon differentiation. The expression of NOD1 and NOD2 increased approximately 50- and twofold, respectively, upon differentiation (Fig. 2B). Osteoblasts were induced to differentiate by the addition of ascorbic acid over a 6 day period using reference index genes ALP and OCN shown in Fig. 3A. In Fig. 3B, osteoblasts are shown to exhibit very similar trends to those of cementoblasts except that the peak periods varied.

Expression of key downstream effector molecules for TLR and NOD signaling in cementoblasts and osteoblasts

In order to begin determining whether cementoblasts express functional TLRs and NODs, we examined whether cementoblasts express downstream effector molecules (1) by RT-PCR. Myeloid differentiation factor 88 (MyD88) is a common adaptor molecule for TLR signaling. Interleukin-1 receptor-associated kinase (IRAK) 4 and Toll/interleukin-1 receptor (TIR) domain-containing adaptor protein (TIRAP) are essential for the MyD88-dependent signaling pathway. Receptor-interacting protein (Rip) 2 kinase is a critical effector molecule in both NOD1- and NOD2-mediated cellular activation (25). Confluent cementoblasts and osteoblasts constitutively expressed the mRNAs for these signaling molecules (Fig. 4).

Activation of NF-κB in cementoblasts upon stimulation with synthetic chemical TLR and NOD ligands

Since most of the TLR and NOD signaling pathways mainly lead to the activation of NF-κB, we examined whether TLRs and NODs expressed by cementoblasts were functional in terms of NF-κB activation upon stimulation with their respective ligands. Pam_3CSK_4 (TLR-1/2 agonist), but not $Pam_2CGDPKHPKSF$ (TLR-2/6 agonist) or lipid A (TLR-4 agonist), significantly activated NF-κB in confluent cementoblasts (day 0 cells; Fig. 5A). Activation of NF-κB was significantly induced upon stimulation with Pam_3CSK_4, $Pam_2CGDPKHPKSF$ and lipid A in day 2 cells (Fig. 5A). In contrast, NF-κB activation was marginal after treatment with CpG DNA (TLR-9 agonist), FK565 (NOD1 agonist) or muramyldipeptide (NOD2 agonist; data not shown). Since TLR-9 and NODs are intracellular receptors, cells were pretreated with cytochalasin D (1 μM) to facilitate stimulation by

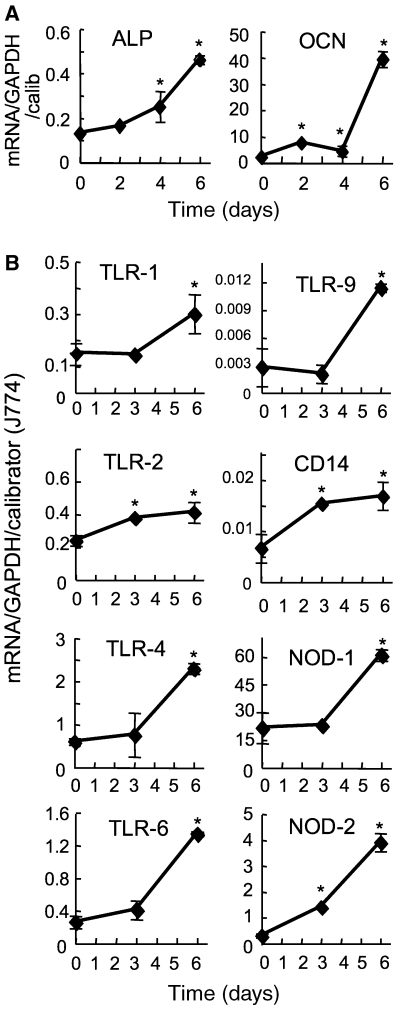

Fig. 3. Expression of TLR-1, -2, -4, -6 and -9, CD14, NOD1 and NOD2 at the gene level in osteoblasts. Confluent MC3T3 cells were cultured in α-MEM containing 5% FBS in the presence of ascorbic acid (50 μg/mL) for the indicated time periods. Total cellular RNA was extracted and the mRNA expression levels were analyzed by real-time quantitative RT-PCR. Representative data of three separate experiments are shown as means ± SD of triplicate assays. Statistical significance is shown (*$p < 0.05$ vs. day 0 control).

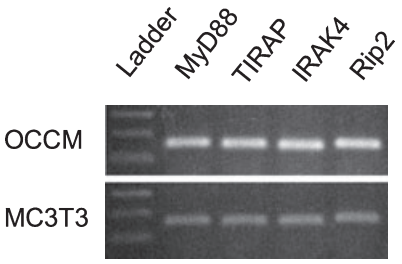

Fig. 4. Expression of key downstream effector molecules for TLR and NOD signaling in cementoblasts and osteoblasts. Total cellular RNA was extracted from confluent OCCM-30 and MC3T3 cells, and the mRNA expression levels of MyD88, TIRAP, IRAK4 and Rip2 kinase were analyzed by RT-PCR. Representative data of three separate experiments are shown.

TLR-9 and NOD ligands (26). It has been well established that cytochalasin D inhibits actin polymerization and allows components to internalize into the cells (27). Activation of NF-κB was detected upon stimulation with FK565, muramyldipeptide and CpG DNA in both day 0 and day 2 cells (Fig. 5B).

Effect of an NF-κB inhibitor on IL-6 production induced by synthetic TLR and NOD ligands

Next, we examined whether TLRs and NODs on cementoblasts are functional in terms of IL-6 production using ELISA. A major function of IL-6, as related to bone, is the induction of osteoclast activity, although it is recognized that IL-6 is a pleiotropic cytokine that has both pro- and anti-inflammatory actions (28). Agonists for TLR-1/2, TLR-2/6 and TLR-4 significantly induced IL-6 production in day 0 cells, and much higher production was observed in day 2 cells (Fig. 6A). Since TLR-9 and NODs are intracellular receptors, day 0 cells were stimulated with these agonists in the presence of cytochalasin D. Significant induction, especially by the NOD2 agonist, was observed for all stimulants (Fig. 6B). When day 2 cells were stimulated in the same way as day 0 cells, much higher production of IL-6 was observed (Fig. 6B). These findings were consistent with the results shown in Figs 1–3, where differentiated cells showed higher expression of TLRs and NODs compared with day 0 cells. All of the inductions in day 0 and day 2 cells were completely inhibited to background levels or lower by pretreatment with 100 μM of PDTC, a NF-κB inhibitor (Fig. 6A,B). These results indicate that cementoblasts express functional TLRs and NODs, the signaling from which is capable of activating NF-κB and leads to IL-6 production.

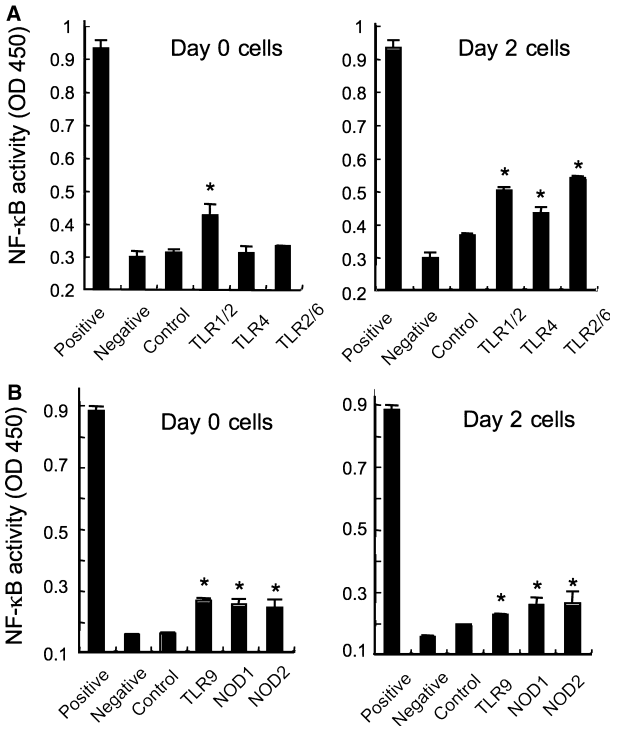

Fig. 5. Activation of NF-κB in cementoblasts triggered by TLR and NOD ligands. Confluent OCCM-30 cells (day 0 cells) were cultured in DMEM containing 5% FBS in the presence of ascorbic acid (50 μg/mL) for 2 days (day 2 cells). (A) Both day 0 and day 2 cells were stimulated with 1 μg/mL Pam_3CSK_4 (TLR-1/2 agonist), 1 μg/mL $Pam_2CGDPKHPKSF$ (TLR-2/6 agonist) or 100 ng/mL lipid A (TLR-4 agonist) for 3 h in DMEM with 5% FBS. (B) Cells were stimulated with 5 μM CpG DNA (TLR-9 agonist), 100 μg/mL FK565 (NOD1 agonist), or 100 μg/mL muramyldipeptide (NOD2 agonist) for 3 h in DMEM with 5% FBS in the presence of cytochalasin D (1 μM) in the same medium to facilitate stimulation by intracellular receptor ligands. All the samples, including the control sample, were adjusted to contain 0.1% (v/v) DMSO in the media during cell culture. After stimulation, whole cell extracts were prepared and a NF-κB ELISA assay was performed. The positive control was provided by a nuclear extract of Jurkat cells. A sample with no cell extract was used as a negative control. Representative data of three separate experiments are shown as means ± SD of triplicate assays. Statistical significance is shown (*$p < 0.05$ vs. day 0 cell control).

Discussion

We have demonstrated that murine cementoblasts as well as osteoblasts express TLR-1, -2, -4, -6 and -9, CD14, NOD1 and NOD2, as well as their adaptor molecules, MyD88, TIRAP, IRAK4 and Rip2 kinase. Furthermore, we showed that the respective ligands induce IL-6 production via NF-κB activation, where CD14 may serve to facilitate ligand recognition by TLR-1/2 as well as TLR-4. These findings suggest that cementoblasts have a similar capacity to osteoblasts to respond to a wide variety of pathogens. However, it has been reported that there are not only similarities but also differences in terms of the reactivity to these ligands. Toll-like receptor-4 signaling triggered by *E. coli* LPS induces production of cytokines, including IL-6, and upregulates RANKL expression in both cell types (17,20,23,29). Toll-like receptor-2 signaling also

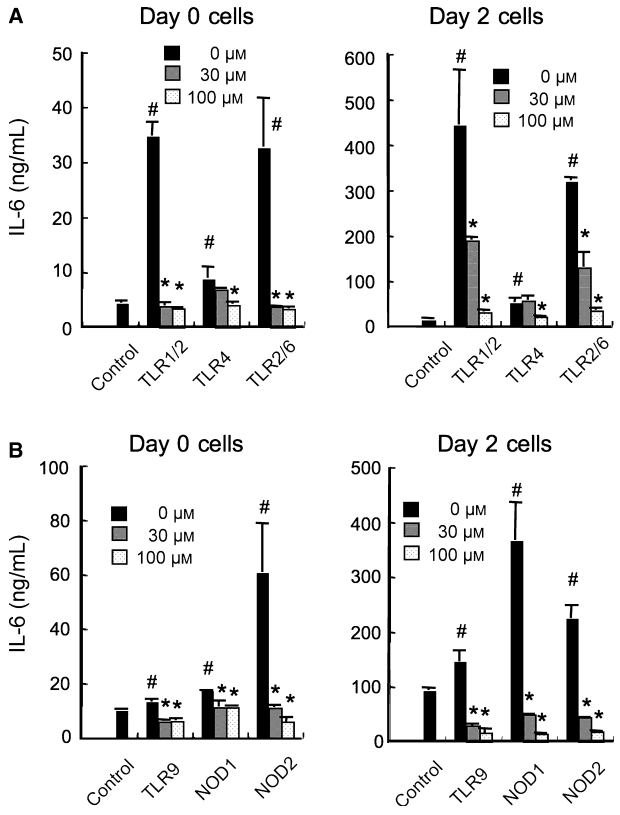

Fig. 6. Interleukin-6 production in cementoblasts triggered by TLR and NOD ligands. Confluent OCCM-30 cells (day 0 cells) were cultured in DMEM containing 5% FBS in the presence of ascorbic acid (50 μg/mL) for 2 days (day 2 cells). Both day 0 and day 2 cells were pretreated with/without the indicated concentrations of PDTC, a NF κB inhibitor, for 1 h. (A) Cells were stimulated with 1 μg/mL Pam3CSK4 (TLR-1/2 agonist), 1 μg/mL Pam2CGDPKHPKSF (TLR-2/6 agonist) or 100 ng/mL lipid A (TLR-4 agonist) in DMEM containing 5% FBS for 12 h. (B) Cells were stimulated with 5 μM CpG DNA (TLR-9 agonist), 100 μg/mL FK565 (NOD1 agonist) or 100 μg/mL muramyldipeptide (NOD2 agonist) in the presence of cytochalasin D (1 μM) in DMEM containing 5% FBS for 24 h. All the samples, including the control sample, were adjusted to contain 0.1% (v/v) DMSO in the media during cell culture. The amount of IL-6 in the supernatants was analyzed by ELISA. Representative data of three separate experiments are shown as means ± SD of triplicate assays. $*p < 0.05$ vs. respective control; $p < 0.05$ vs. control culture.

upregulates RANKL in both cell types (23,30). In contrast, OPG, which is downregulated by *E. coli* LPS in osteoblasts (17,20), is constitutively expressed and not altered in cementoblasts under the same stimulation conditions (23), suggesting that cementoblasts, unlike osteoblasts, may have inhibitory properties for osteoclastogenesis generated by the innate immunity response. This notion is consistent with the clinical observation that root resorption is rarely linked to periodontal disease, even at a stage of severe disease with marked resorption of alveolar bone. In periapical inflammatory lesions, bone resorption can usually be observed rather than external periapical root resorption, although periforaminal resorption has been associated with it (31).

We demonstrated that the expressions of TLRs and NODs were upregulated upon differentiation in both cementoblasts and osteoblasts in a similar manner; however, gene expression levels over 5 days of differentiation were quite different. The OCCM-30 cell line was established based on *in situ* hybridization data, i.e. only cementoblasts expressed strong bone sialoprotein (BSP) and OCN gene signals, while PDL cells did not (24). The OCCM-30 cell line is considered as a mature cell line because it expresses high levels of BSP and OCN transcripts. In contrast, precursor cell lines, such as MC3T3-E1 cells, require additional factors to initiate the expression of BSP and OCN. The OCCM-30 (24) and MC3T3-E1 cell lines (32) require several days and 2 weeks, respectively, before mineralized nodules are formed in the presence of ascorbic acid *in vitro*. Therefore, the different gene expression levels are probably due to the different maturation stage of the cells.

It has been reported that the expression levels of TLR-2 (16,17), NOD1 and NOD2 (19,20) mRNAs in mouse osteoblasts are increased by *E. coli* LPS and exposure to bacteria. Since the promoter regions of these genes possess NF-κB binding site(s) (33,34), activation of NF-κB has been implicated in the induction of mouse TLR-2 and NOD2 expression. In this study, we showed that the expression of TLRs and NODs was upregulated upon differentiation induced by ascorbic acid. However, NF-κB does not seem to be involved in this regulation, since ascorbic acid was reported to inhibit NF-κB activation (35).

Another question that arises is, why do cementoblasts and osteoblasts, which do not even reside in the first line in bacterial challenge, need to amplify PRR expression in the course of cell differentiation? Recent evidence suggests that TLR-2 and TLR-4 are involved in the recognition not only of microbes but also of endogenous ligands, including heat shock proteins (36,37) and extracellular matrix components, such as fibronection (38), soluble hyaluronic acid (39), heparan sulfate (40) and biglycan (41). Therefore, upregulation of TLRs during cell differentiation, while speculative at this point, might be involved in the recognition of endogenous ligands.

In conclusion, we report for the first time that cementoblasts functionally express a wide range of PRRs, and the upregulation of these receptors accompanies cell differentiation. The alteration of cell function mediated by TLR/NOD signaling needs to be explored further in order for us to understand the role of cementoblasts in

inflammation/regeneration in periodontal tissue.

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