[Frontiers in Bioscience 13, 7064-7071, May 1, 2008]

Notch is activated in RANKL-induced osteoclast differentiation and resorption

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1. ABSTRACT

The process of osteoclast differentiation and resorption is fine-tuned by signal pathways, which need to be further elucidated. The aim of this study was to explore the possible connections between NF-κB and Notch in RANKL-induced osteoclast activity. To this end, RANKL was used to stimulate mouse osteoclast precursor cell line RAW264.7. The number of multinucleated $TRAP^+$ osteoclasts was counted and the resorption area was measured. NF-κB transcriptional factor activity was determined by EMSA. Quantitative RT-PCR and Western blotting analysis were used to determine Hes1 (one of Notch signaling primary targets) mRNA and protein expressions respectively. Mature osteoclasts and bone resorption areas were detected in the present study. NF-κB activity was increased in RANKL-induced osteoclast differentiation and resorption. mRNA and protein expressions of Hes1 in RAW264.7 cells were up-regulated after RANKL stimulation. In conclusion, NF-κB signaling mediated RANKL-induced osteoclast differentiation and resorption, during which, Notch signaling was activated. Therefore, Notch could be a novel therapeutic target for bone resorption related diseases.

2. INTRODUCTION

Bone remodeling is essential for the regulation and maintenance of skeletal integrity, which depends on a delicate balance between bone formation and bone resorption. Bone-forming osteoblasts and bone-resorbing osteoclasts play central roles in bone remodeling (1). Stimulation of local bone remodeling around teeth, such as during orthodontic treatment, results in tooth movement (2, 3). Tipping the balance of bone remodeling in favor of osteoclasts leads to pathological bone resorption, as seen in bone disease i.e. periodontitis and autoimmune arthritis (4, 5).

Osteoclasts are large multinucleated cells that derived from hematopoietic precursor cells. Under specific microenvironment, preosteoclasts differentiate into mature osteoclasts. A number of systemic and local factors are known to influence osteoclast formation and function. These include interleukin-1 (IL-1), IL-6, IL-11, tumor necrosis factor-alpha and TNF-beta (6, 7). Recently, an essential signaling system for cytokines in osteoclast biology has been identified. This system involves receptor activator of NF-kappa B ligand (RANKL)/ RANK/

osteoprotegrin (OPG) (8-10). Furthermore, RANKL/OPG system is involved in bone remodeling and under the control of nearly all osteotrophic hormones and local pro-resorptive factors produced in the bone microenvironment (11, 12). The binding of RANKL to its receptor RANK, which is expressed by osteoclast precursors, activates several intracellular pathways such as nuclear factor kappa B (NF-κB), nuclear factor of activated T-cell (NFAT) (13-16). The transduction of the RANKL signal results in fusion of mononuclear preosteoclasts into multinucleated osteoclasts.

Increasing evidences have demonstrated the critical role of RANKL/RANK signaling in osteoclast differentiation and its cross-regulation with other signaling molecules fine-tunes this process (17). Notch is an evolutionarily conserved cell signaling pathway that participates in multiple cellular processes: differentiation, proliferation, adhesion, migration and apoptosis. Moreover, it becomes evident that the outcome of Notch signaling is strictly context-dependent and differences in the strength, timing, cell type and context of the signal may all affect the final outcome (18). Some connections between NF-κB and Notch signaling have been demonstrated by previous studies (19, 20). However, the role of Notch in osteoclast activity has not received much attention. Minor knowledge is available about the effects of RANKL on Notch signaling in bone remodeling. It is essential to understand how Notch integrates inputs from other signaling pathways, since this knowledge may give clues for future therapeutic applications. The aim of the present study was to investigate the possible involvement of Notch signaling in RANKL-induced osteoclast activity.

3. MATERIALS AND METHODS

3.1. Cell cultures

The murine monocytic cell line RAW264.7 (ATCC, VA, USA) were cultured in RPMI1640 (BioWhittaker, Verviers, Belgium) and 10% fetal bovine serum (FBS). All media were supplemented with 2 mM glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Incubations were performed at 37°C in 5% humidified air. For osteoclast differentiation and all other experiments in this study, alpha-MEM medium was used.

3.2. Osteoclast differentiation assay

Cells were plated at a concentration of $1.3\times10^4/cm^2$ into chamber slides in the presence of RANKL (100 ng/ml) (Antigenix, NY, USA). Medium was changed every 2 days. After 5 days of culture, the cells were fixed and stained using the TRAP staining kit (Sigma-Aldrich, Munich, Germany) according to the manufacturer's instructions. TRAP-positive cells with more than three nuclei were considered to be osteoclast-like cells. The number of osteoclast-like cells was counted under light microscope.

3.3. Resorption assay

Cells were plated at a concentration of $1.3\times10^4/cm^2$ onto Osteoclast Activity Assay Substrate (OCT inc., Choongnam, Korea) in the presence of RANKL (100 ng/ml). Medium was changed every 2 days. After 10 days of culture, the plates were washed in 6% sodium hypochloride solution to remove the cells. The resorption areas on the plates were photographed with a digital camera attached to the microscope and analyzed by an Image Analysis System (Leica, Solms, Germany).

3.4. Quantitative RT-PCR

Cells were plated at a concentration of $1.3\times10^4/cm^2$ in 60 mm tissue culture dishes in the presence of RANKL (100 ng/ml). After 24 h of incubation, total RNA was extracted using Absolutely RNA Miniprep kit (Stratagene, CA, USA) according to the manufacturer's instructions. cDNA was synthesized with superscript reverse transcriptase (Invitrogen, Nelson, UK), followed by quantitative real time PCR with gene specific primers. Hes1 primers (sense, 5-ACCCCAGCCAGTGTCAACA-3; antisense, 5'-CATGATAGGCTTTGATGACTTTCTG-3', 78bp, GenBank accession number AK133538) were designed using Primer Express software (Applied Biosystems, CA, USA). For β-actin, the sequences used were: sense, 5'-CTGAACCCTAAGGCCAACCGTG-3'; antisense, 5'-GGCATACAGGGACAGCACAGCC-3' (105bp, GenBank accession number NM007393). Relative quantities of mRNA expression were analyzed using quantitative RT-PCR (Bio-Rad, CA, USA) in the presence of SYBR Green (Applied Biosystems, CA, USA). For the normalization of each sample, the expression level of the gene was divided by that of beta-actin.

3.5. Cell extracts preparation

Cells were plated at a concentration of $1.3\times10^4/cm^2$ in 60 mm tissue culture dishes in the presence of RANKL (100 ng/ml). After 48 h of incubation, adherent cells were washed twice with ice-cold PBS, scraped off the dishes, and collected by centrifugation. Whole cell lysates were prepared with TNE buffer (10mM Tris-HCL, pH7.8, 0.15 M NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 mM DTT and protease inhibitor cocktail). To prepare cytosolic and nuclear proteins, cell pellets were homogenized with cytoplasmic lysis buffer. The tubes were gently vortexed for 15 min. Nuclei were collected by centrifugation at 14,000 g for 1 min, and the supernatants were stored at -80 °C. To obtain nuclear protein extracts, the pellets were resuspended in complete lysis buffer, and gently shaken for 30 min at 4 °C, followed by centrifugation at 14,000 g for 10 min. Protein concentrations of the extracts were determined by the Bio-Rad protein assay agent (Bio-Rad, Muchen, Germany).

3.6. Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed using NF-κB oligonucleotide probe (5'-AGTTGAGGGGACTTTCCCAGGC-3') end-labeled with IRDye 700 (Biolegio, Nijmegen, The Netherlands). Nuclear protein (2 µg) was pre-incubated for 15 min at room temperature in binding buffer, consisting of 10 mM Tris–Cl, pH 7.5, 1 mM $MgCl_2$, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 4% glycerol, and 0.05 µg/ml poly (deoxyinosinic deoxycytidylic acid). After addition of 2 µl IRDye 700-labeled oligonuleotide probe, the incubation was continued for 30 min at room temperature. The

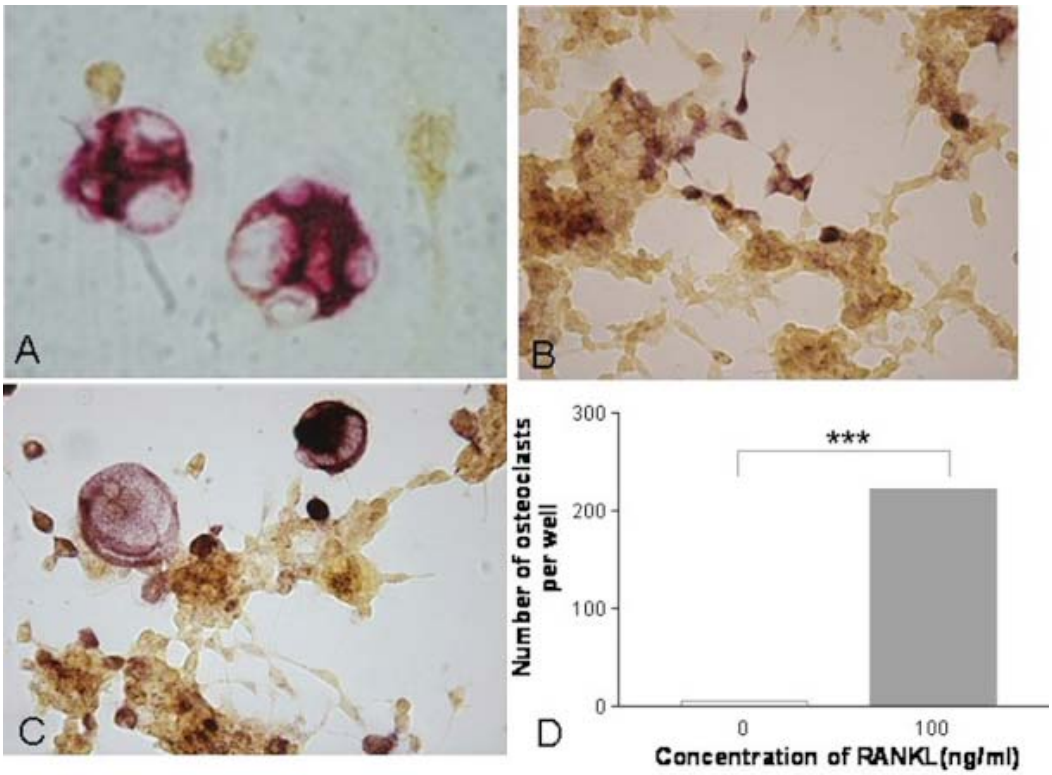

Figure 1. Osteoclastogenesis in RAW264.7 cells induced by RANKL. The cells were cultured then stained for TRAP. The TRAP-positive multinucleated cells containing more than three nuclei were counted as osteoclasts. (A) Typical morphology of osteoclasts is multinucleated cells with TRAP-positive staining. Cytoplasm is wine-red stained while nuclei without staining. Magnification 100×. (B) RAW264.7 cells were cultured 5 days without RANKL stimulation then stained for TRAP. Magnification 40×. (C) RAW264.7 cells were cultured 5 days with 100ng/ml RANKL stimulation then stained for TRAP. Magnification 40×. (D) Number of osteoclasts after 5 days of culture. The data are the means ± SD for the cultures. Statistical analysis was performed by student's *t*-test. ***P<0.001.

reaction was terminated by adding 1 μl gel loading buffer, and the mixture was subjected to non-denaturing 4% polyacrylamide gel electrophoresis in 0.5 × TBE buffer. The gel was scanned by Odyssey Infrared Imager (Li-Cor Biosciences, Nebraska, USA), and the bands were quantified by Quantity One using Image Quant software (Bio-Rad, CA, USA).

3.7. Western blotting analysis

Fifteen micrograms of protein was resolved on a 10% SDS-PAGE gel, transferred to a nitrocellulose membrane (Millipore, MA, USA), and probed with primary antibody Hes1 and beta-actin (Santa Cruz Biotechnology Inc., CA, USA). After incubation with species-specific HRP-conjugated secondary antibodies (Dako, Glostrup, Denmark) for 15 min, the ECL Western blotting detection system (Roche Diagnostics, Mannheim, Germany) was applied. The chemiluminescence was detected and quantified by Quantity One software (Bio-Rad, CA, USA).

3.8. Statistical analysis

All data were normally distributed. Student's t-test or ANOVA tests were used to compare 2 or more than 2 groups. Differences were considered significant when $p < 0.05$ (two-tailed).

4. RESULTS

4.1. RANKL stimulated osteoclast-like cell formation of RAW264.7

To confirm the effect of RANKL on osteoclastogenesis, RAW264.7 cells, incubated in differentiation medium, were exposed to 100 ng/ml of RANKL. Osteoclasts are TRAP-positive multinucleated cells possessing more than three nuclei (Figure 1A). In the absence of RANKL, the number of TRAP positive multinucleated cells was 6.17 ± 2.04 (Figure 1B). In contrast, the number of osteoclasts was 222.83 ± 21.18 in the presence of RANKL (Figure 1C). Addition of RANKL significantly increased the number of TRAP-positive multinucleated cells (P<0.001) (Figure 1D).

4.2. RANKL induced significant bone resorption in RAW264.7 cells

Since the ability of RANKL to stimulate the formation of osteoclast-like multinucleated cells has been confirmed, further experiment was performed to examine the effect of RANKL on osteoclastic bone resorption. Calcium phosphate-coated culture plates were used to detect the bone resorption ability of mature osteoclasts. Significant osteoclast formation was not identified in

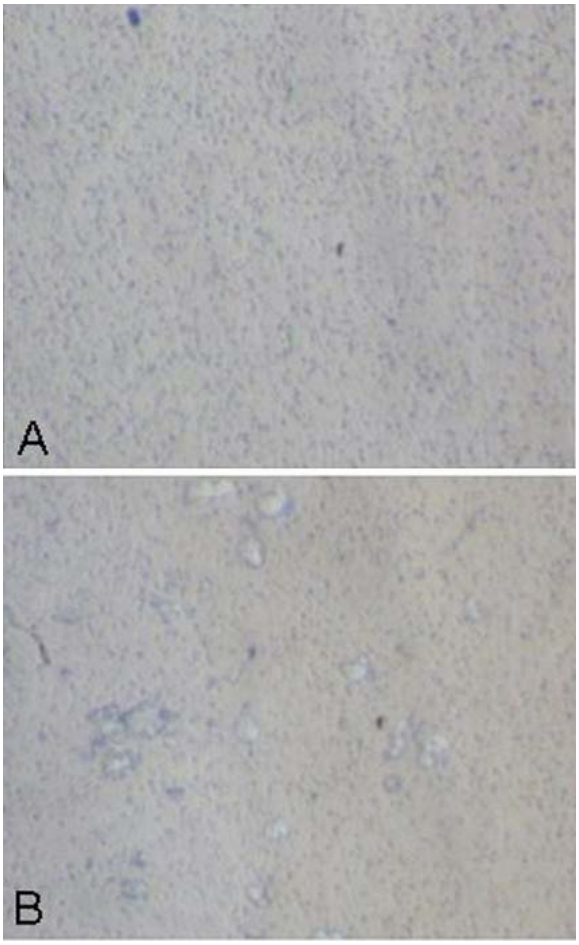

Figure 2. RANKL stimulated bone resorption of RAW264.7 cells. Demonstration of pit formation on an Osteoclast Activity Assay Substrate plate using RAW264.7 cells stimulated with 100ng/ml RANKL after 10 days of culture. (A) Without RANKL stimulation. Magnification 5.0×3.6. (B) With 100ng/ml RANKL stimulation. Magnification 5.0×3.6.

cultures incubated in medium without RANKL (Figure 2A). However, RANKL-stimulated RAW264.7 cells showed a number of resorption areas (Figure 2B). Addition of RANKL exerted strong effect on bone resorption ability of RAW264.7 cells.

4.3. RANKL stimulated the activation of NF-κB in RAW264.7 cells

The interaction of RANKL with RANK leads to activation of the signaling pathways including NF-κB. In the present study, NF-κB DNA binding activity was detected with EMSA. First, the NF-κB probe specificity was tested with competitive analysis (Figure 3A). Shift bands decreased with increasing amount of unlabelled NF-κB while no change of shift band with addition of non-competitive Sp1 probe. Treatment of RAW264.7 cells with RANKL increased NF-κB DNA binding activity compared with the controls (Figure 3B, 3C). Taken together, these data confirmed RANKL can activate NF-κB signaling in RAW264.7 cells.

4.4. RANKL up-regulated Notch-dependent Hes1 expression in RAW264.7 cells

In some cell context, NF-κB signaling regulates Notch-dependent Hes1 expression leve1 (21). Here, we examined Hes1 expression by RT-PCR and western blotting analysis after NF-κB activation in RANKL-stimulated RAW264.7 cells. Without RANKL stimulation, mRNA of Hes1 was 0.23 ± 0.04. After RANKL treatment, mRNA of Hes1 was 0.49 ± 0.05. Treatment of RAW264.7 cells with RANKL significantly up-regulated the Hes1 mRNA expression level compared with the controls ($p<0.001$) (Figure 4A). Results of western blotting showed RANKL increased the Hes1 band density of RAW264.7 cells (Figure 4B, 4C).

5. DISCUSSION

Derived from hematopoietic precursor cells, osteoclast is the pivotal cell in the degradation of bone matrix (22). Recent studies have suggested a crucial role of RANKL in osteoclast differentiation and bone resorption (17). Also novel insights suggest that Notch could be involved in osteoclast activities since Notch signaling is implicated in the development of hematopoietic cells (23, 24). However, up to now, the association between RANKL and Notch signaling in osteoclast activity has not been studied. Findings of the present study demonstrated that NF-κB was activated in RANKL-induced osteoclast differentiation and bone resorption. And for the first time our study showed a positive correlation between RANKL-induced NF-κB activation and up-regulation of Notch-dependent Hes1 gene expression in osteoclast activity.

Previous studies have suggested that RANKL acts as a positive regulator in osteoclastgogenesis and bone resorption (25-28). Overproduction of RANKL is implicated in a variety of degenerative bone diseases, such as rheumatoid arthritis, periodontitis (29, 30). mRNA of RANKL is expressed in periodontal ligament cells, osteoblasts, osteoclasts, stromal cells, T lymphocytes, endothelial cells and epithelial cells (25, 31-36). Two recent studies have shown that activated T and B lymphocytes were the major sources of RANKL protein in periodontally diseased gingival tissues (33, 34). Inflammatory cytokines including IL-1, TNF-alpha and prostaglandin E_2 (PGE_2) induce RANKL expression (7, 37-40). During inflammatory processes in periodontal disease, IL-1, TNF-alpha and PGE_2 are major cytokines produced at diseased sites and are involved in the alveolar bone resorption (33, 34). In the present study, direct stimulation with RANKL resulted in osteoclast formation and bone resorption in RAW264.7 cells, thus providing further evidence that RANKL is a positive osteoclastogenic stimulator and plays a crucial role in bone resorption.

The mechanism of RANKL-induced osteoclast differentiation and bone resorption has been previously studied. Several transcriptional factors have been implicated in RANKL-RANK interaction including NF-κB

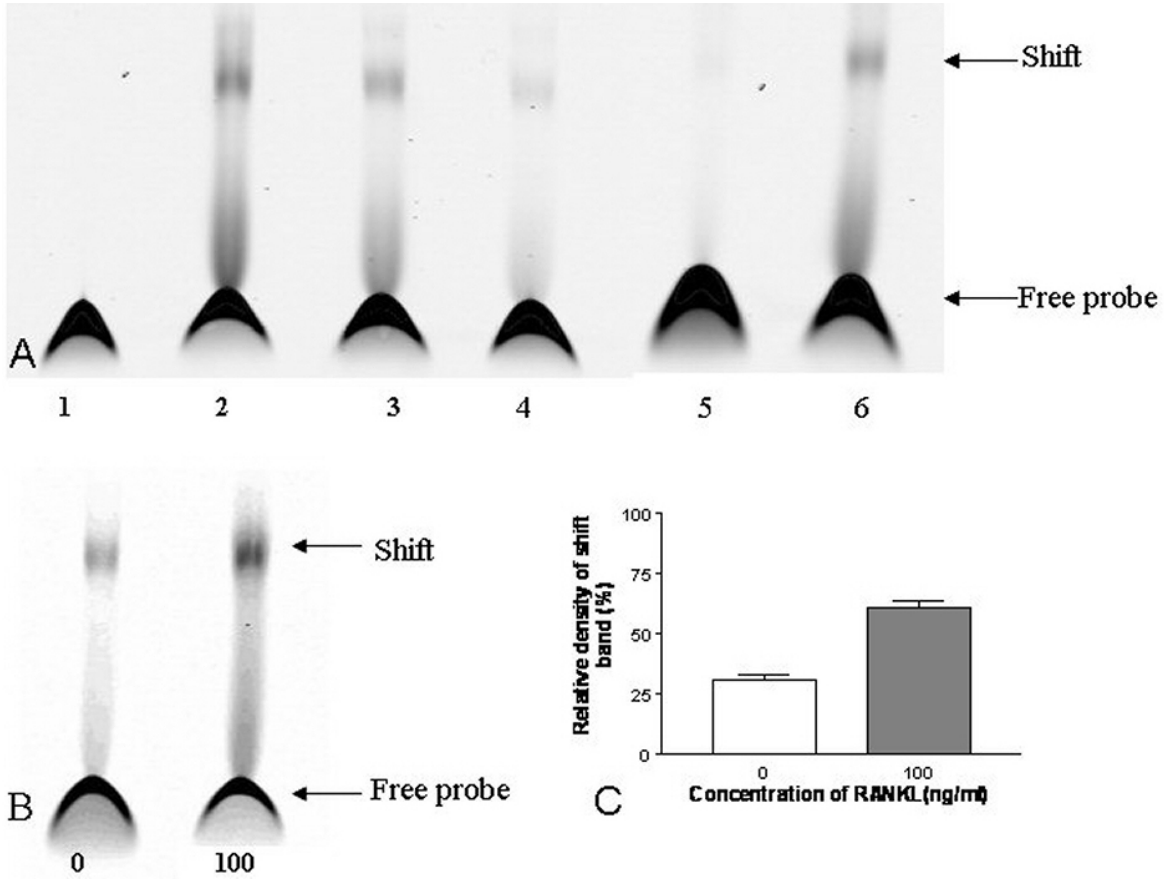

Figure 3. RANKL increased NF-κB DNA binding activity. **(**A) Competitive analysis. Nuclear extracts were prepared from RAW264.7 cells and subjected to analysis for NF-κB DNA binding activity, as measured by EMSA. (1) without nuclear extracts; (2) nuclear extracts and IRDye700 labelled NF-κB probe; (3) nuclear extracts and IRDye700 labelled NF-κB probe in the presence of 50× unlabelled NF-κB probe; (4) nuclear extracts and IRDye700 labelled NF-κB probe in the presence of 100× unlabelled NF-κB; (5) nuclear extracts and IRDye700 labelled NF-κB probe in the presence of 200× unlabelled NF-κB; (6) nuclear extracts and IRDye700 labelled NF-κB probe in the presence of 200× unlabelled Sp1 probe. (B) Nuclear extracts were prepared from RAW264.7 cells with or without RANKL stimulation. The DNA binding activity was measured. (C) Densitometric quantification of the bands of Western blotting is shown. The histogram indicates the relative band intensity, in arbitrary densitometric units, derived from densitometric scans of 3 independent experiments. Results are expressed as percentage of NF-κB shift band density to that of free probe.

(13-16). NF-κB is activated in response to the stimulation of a variety of cell-surface receptors. This involves translocation of NF-κB from the cytoplasm to the nucleus, where it functions as a transcription factor (41-43). These previous studies suggest that any stimulator resulting in RANKL upregulation can lead to the formation and activation of osteoclasts in the alveolar bone. A recent study (44) reported that transactivation of NF-κB occurred in response to RANKL treatment in mouse marrow osteoclasts. Another study (3) suggested one component of NF-κB, p65 levels were increased by orthodontic stimuli. This may be a mechanism through which NF-κB can be rapidly activated. The fact that increased NF-κB transcriptional activity was detected in RAW264.7 cells in response to RANKL supports this mechanism.

The most important finding of the present study is the up-regulation of Notch-dependent gene Hes1 resulting from RANKL-induced NF-κB activation in RAW264.7 cells. Notch is a single-pass transmembrane receptor that is activated by direct contact with the membrane-bound ligands Delta 1-4 and Serrate/Jagged 1 and 2. Receptor-ligand interaction is followed by two successive cleavage events. The result of these two cleavages is the release of the soluble intracellular domain of Notch (ICN), which translocates into the nucleus, where it interacts with other elements and induces expression of target genes. The best-known Notch target genes are members of the basic helix-loop-helix (bHLH) hairy/enhancer of split (Hes) family and the Herp (Hes-related repressor protein) family (45). Extensive literature supported that Hes1 is a primary Notch target (46). Numerous studies have demonstrated a role for Notch in hematopoietic stem cells and tumor cellular fate (22, 47). Although Notch signaling in stem cells and tumor development is largely understood, its role in bone

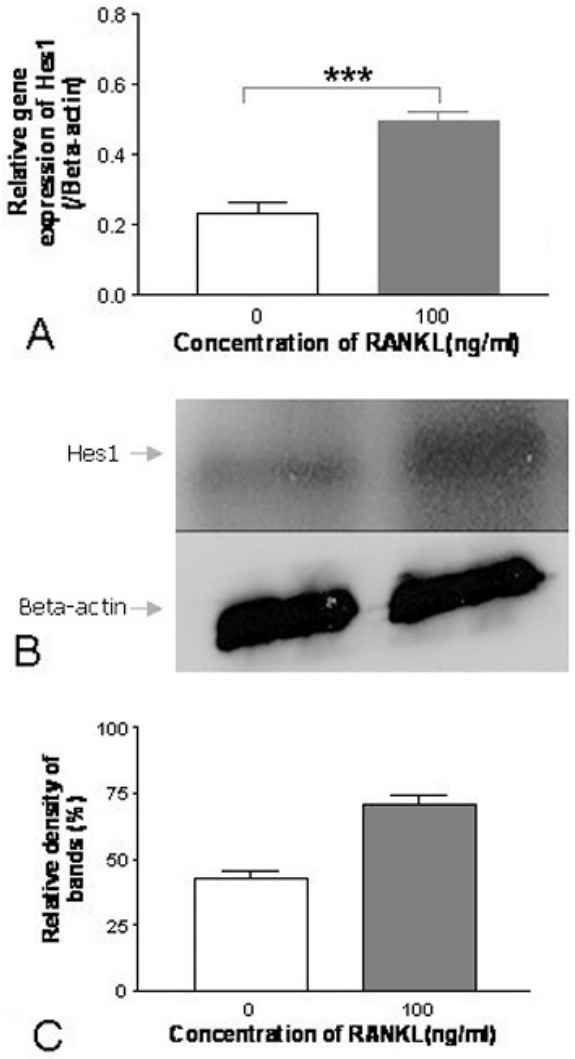

Figure 4. RANKL up-regulated Hes1 expression in RAW264.7 cells. (A) 24 h after RANKL stimulation, cells were harvested and the expression of Hes1 mRNA was examined with real time PCR. The results were expressed as the ratio of Hes1 to actin. Values presented are the means ± SD of three replicate meansurements. ***P<0.001 (B) 48 h after RANKL stimulation, whole cell lysates were prepared, and equal amount of proteins (15 μg) were analyzed by Western blotting using antibody to Hes1 and beta-actin. (C) The intensity of the bands was determined by densitometry and normalized with beta-actin. Values shown are means ± SD of three independent experiments.

remodeling is poorly known. Evidence from a previous study (48) demonstrated that Notch inhibited osteoclast differentiation. Our study demonstrated that NF-κB activation leads to the activation of Notch signaling pathway in osteoclast activity, which suggests a possible cross-regulation between Notch and NF-κB.

In conclusion, the present study confirmed RANKL acts as the crucial factor to induce osteoclast differentiation, and for the first time, provided evidence that Notch signaling plays a role in RANKL-induced osteoclast formation and function. Moreover, RANKL-induced NF-κB activation resulted in up-regulation of Notch-dependent gene Hes1. A cross-regulation of NF-κB and Notch is suggested which might be a novel therapeutic target for bone resorption related diseases.

6. ACKNOWLEDGEMENTS

This study as supported by an NWO Research Grant (no. 016 056 171) from Dutch Scientific Organization and an EOS Research Grant from European Orthodontic Society.

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Abbreviations: NF-κB: nuclear factor kappa B; RANKL: nuclear factor kappa B ligand; RANK: receptor activator of NF kappa B; OPG: osteoprotegrin; EMSA: electrophoretic mobility shift assay; RT-PCR: real-time polymerase chain reaction; TRAP: tartrate resistant acid phosphatase; NFAT: nuclear factor of activated T-cell; IL: interleukin; PGE 2: prostaglandin E 2; ICN: intracellular domain of Notch; bHLH: basic helix-loop-helix; Hes: hairy and enhancer of split; Herp: Hes-related repressor protein

Key Words: RANKL, Osteoclast, Notch, NF-κB, Hes1, Bone Resorption

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