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RANK-L induces the expression of NFATc1, but not of NFκB subunits during osteoclast formation

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Abstract

We report the program of gene expression during osteoclast formation from RAW264.7 cell precursors in response to RANK-ligand (RANK-L) using a combination of quantitative real time PCR and Affymetrix gene chip assays. We found that genes obligatory to osteoclast formation and function, namely tartrate-resistant acid phosphatase, cathepsin K, β3 integrin, and calcitonin receptors, were up-regulated by RANK-L markedly by up to ~2000-fold. In contrast, we found a cluster of genes that were significantly down-regulated: these included interleukin-18, insulin-like growth factor-1, interleukin-6 receptor, and cathepsins B, C, and L. These results from real time PCR were broadly concordant with those obtained from Affymetrix. We also explored the expression of the transcription factors of the NFAT and NFκB family at days 3 and 5 of culture. Whereas NFATc1 expression was increased significantly at days 3 and 5 following RANK-L exposure, there were no significant increases in the expression of NFκB subunits, namely p65, p50, c-Rel, IκBα, and IκBβ. There were also no significant differences in transcription modulator expression between days 3 and 5, except for c-*Rel* and NFATc4, which were both decreased significantly at day 5. The studies suggest RANK-L regulates the expression only of NFATc1, while it signals through both NFATc1 and NFκB.

Keywords: Osteoclast; NFATc; Microarray; NFκB

The osteoclast is a cell unique in its ability to resorb bone. It is formed from the hematopoetic stem cell precursor, mainly under the control of the osteoclastogenic cytokine, receptor activator for NFκB-ligand (RANK-L) that interacts with the RANK receptor on the hematopoetic stem cell precursor [1]. Two key transcription modulators have been identified that are both RANK-L-sensitive. One of these, the NFκB system, is activated upon the phosphorylation of its inhibitory component IκB that dissociates from heterodimeric complexes between the subunits p65, p50, and c-Rel [2]. A more recently described transcription regulator is nuclear factor for activated T cells (NFAT) [3,4]. NFATs are dephosphorylated by the heterodimeric Ca^{2+}/calmodulin phosphatase, calcineurin; this permits their nuclear translocation [5]. NFAT has four isoforms, c1–c4, each with a highly conserved DNA binding domain. These isoforms regulate the differentiation and function of many different cell types [6,7]. The c1 isoform is critical for cardiac valve and skeletal myofiber formation rendering $c1^{-/-}$ mice embryonic lethal. NFATc2 regulates vascular endothelial cell, skeletal myotube, chondrocyte, adipocyte, and pancreatic acinar cell differentiation. Likewise, NFATc3 and c4 control perivascular tissue and keratinocyte development. Recent studies have suggested that NFATc1 is a master switch for osteoclastogenesis in response to RANK receptor activation [3,4,8].

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RANK-L stimulates the nuclear translocation of NFκB subunits within minutes [9] and the translocation of NFATc1 within 24–48 h [3,4]. We utilized real time quantitative PCR to examine the expression of a number of osteoclastic genes, namely TRAP, cathepsin K (and other cathepsins), metalloproteinase-9 (MMP-9), integrin β_3, calcitonin receptor, and IL-6 receptor, following a 5-day culture of osteoclast precursor RAW264.7 cells treated with RANK-L. RNA obtained from these cultures was also subjected to DNA Affymetrix screening. We next utilized the real time technique to quantify the expression of the NFκB subunits p65, p50, and c-Rel, IκBα and IκBβ, NFAT isoforms, and the enzyme calcineurin in day 3 and day 5 RAW264.7 cell cultures in response to RANK-L.

We established concordance between results obtained from real time PCR and Affymetrix chip assays. More importantly, we found that whereas the expression of NFκB components was not altered by RANK-L treatment, there was a 30-fold elevation of NFATc1 mRNA during osteoclastogenesis. This suggests that RANK-L both controls the nuclear translocation of NFATc1 by activating calcineurin and regulates, in the longer term, expression of this critical osteoclastogenic transcription factor.

Materials and methods

We examined gene expression in RAW264.7 cells that were cultured in RANK-L (60 ng/ml) for up to 5 days in α-MEM and fetal bovine serum (10% v/v). RAW264.7 is a mouse macrophage cell line that, in the presence of RANK-L, differentiates into tartrate-resistant acid phosphatase (TRAP) positive, multinucleated, bone-resorbing osteoclasts [1,10]. RAW264.7 cells thus represent an excellent and well-characterized model for studying osteoclastogenesis in vitro in the absence of contaminating osteoblasts. Cells were harvested after 3 and 5 days of culture with RANK-L. Three-day cultures had TRAP-positive mono- and multi-nucleated osteoclasts as well as precursor cells, while at day 5, the population became predominantly osteoclastic. Total RNA was extracted using a StrataPrep Total RNA miniprep kit (Stratagene, La Jolla, CA), per manufacturer's protocol. Expression levels of various transcripts were determined by quantitative real time RT-PCR.

For real time PCR, 5 μg total RNA was first reverse transcribed (RT) into cDNA. 1/200 (approximately 500 pg) RT mixture was utilized for 40 cycle three-step PCR in an ABI Prism 7900HT (Applied Biosystems, Foster City, CA) in 20 mM Tris, pH 8.4, 50 mM KCl, 5 mM $MgCl_2$, 200 μM dNTPs, 0.5× SYBR Green I (Molecular Probes, Eugene, OR), 200 nM each primer, and 0.5 U Platinum *Taq* DNA polymerase (Invitrogen).

The cumulative fluorescence for each amplicon was normalized to that seen with GAPDH amplification. The target signal was plotted against the number of PCR cycles, and comparisons between samples were made at the point where all the sample reactions were in the linear phase of amplification (the crossing threshold). The intersection of each amplification curve with the threshold yielded a C_T (threshold cycle) value that reflected the relative amount of the original mRNA and cDNA (Fig. 1A). The more the initial transcript, the lower the C_T value. The chosen primers were designed to yield close to 100% amplification efficiency; thus, each amplification cycle led to a doubling of amplified fragment. A C_T of 20 represented twice as much starting mRNA as a C_T of 21. The initial normalized value in each sample was represented by the ΔC_T (target C_T − GAPDH C_T). The results were then calculated as the difference between each normalized reporter signal in a treated sample versus that in control tubes, yielding the $\Delta\Delta C_T$ (treatment ΔC_T − control ΔC_T) [11]. Results were expressed as the fold-increase, at each time point, over the respective GAPDH controls (calculated as $2_T^{\Delta\Delta C}$). Means (± SEMs) were calculated from pooled data from up to three separate experiments, each with triplicate replicates.

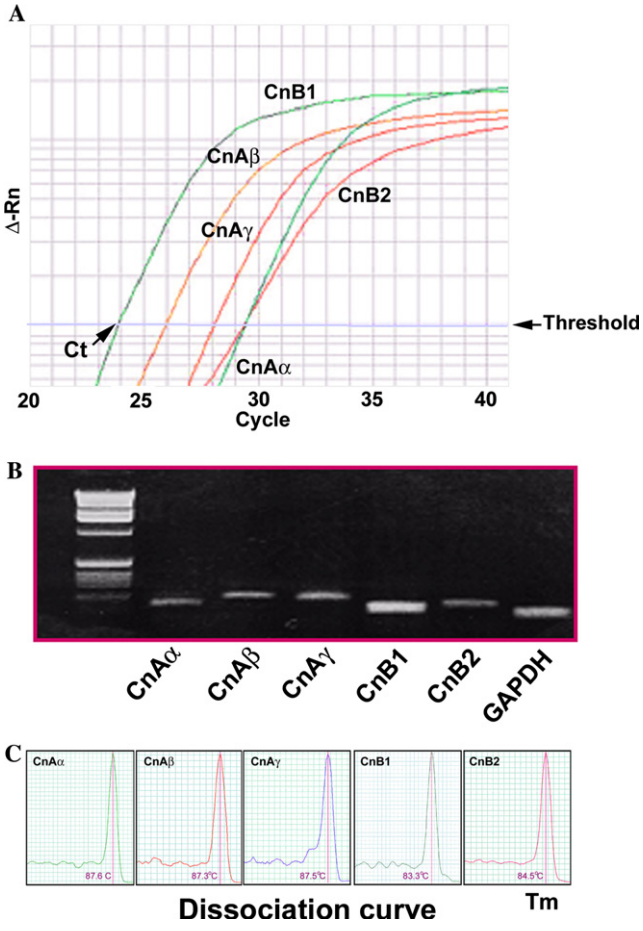

Fig. 1. (A) Amplification curves for the calcineurins, calcineurin (Cn) Aα, Aβ, Aγ, B1, and B2 with the C_T (threshold cycle) value that reflects the relative amount of the original mRNA and cDNA. The more the initial transcript, the lower the C_T value. Δ-R_n (baseline-subtracted normalized reporter) is shown on the left. T_m, melting temperature. (B) Single bands of the expected size for each PCR amplicon demonstrated by agarose gel electrophoresis. Lane 1, DNA marker. (C) Single sharp peaks showing melting temperatures for each set of PCR primers (as indicated) as part of a routine association analysis to check for primer quality.

The method was validated by demonstrating a single band of the expected size for each PCR amplicon by agarose gel electrophoresis (Fig. 1B). Primer quality was checked routinely by obtaining single sharp peaks upon melting point association analysis (Fig. 1C). These tests established the specificity of each PCR, enabling accurate mRNA quantitation.

In separate, but parallel experiments, we performed microarray gene screening to determine whether results from real time PCR were concordant. cDNA was prepared from duplicate RNA extracts of cells

harvested at day 5 that were treated with either RANK-L (60 ng/ml) or vehicle. These were then hybridized to Affymetrix u74Av2 mouse genome arrays.

Results and discussion

This study explores in depth the expression of a variety of osteoclast regulatory and other genes during osteoclast development using the model RAW264.7 cell system that is sensitive to differentiation by RANK-L. Our objectives were to: (a) evaluate whether changes in gene expression measured by real time PCR and Affymetrix assays after a 5-day culture with RANK-L were concordant, and (b) detect if there were significant differences in gene expression between 3 and 5 days of culture.

Table 1A shows that markers of osteoclast differentiation, namely TRAP, cathepsin K, MMP-9, and the calcitonin receptor, showed an increase of several hundred-fold at day 5 with real time PCR. Overall concordance was noted with the Affymetrix chips, but in some cases, prominently with cathepsin K and TRAP, the two technologies yielded different relative increases. β_3 integrin was relatively less elevated at day 5, as was NFATc1. Also impressive was a concordance between measurements in the expression of genes that were down-regulated during osteoclastogenesis (Table 1B).

Table 1
RANK-L-induced changes in the expression of various genes, including tartrate-resistant acid phosphatase (TRAP), cathepsin K, metalloproteinase 9 (MMP-9), calcitonin receptor, β_3 integrin, nuclear factor for activated T cells c1 (NFATc1) (A), interleukin 18, insulin-like growth factor-1 (IGF-1), cathepsins L, C, and B, and interleukin 6 receptor (B) during osteoclastogenesis from RAW264.7 cells

	Real-time PCR	Affymetrix
(A) Up-regulated molecule		
TRAP	+1184	+29
Cathepsin K	+582	+13.9
MMP-9	+530	+408
Calcitonin receptor	+139	+76.1
Integrin β_3	+19.6	+8.4
NFATc1	+12.8	+6.2
(B) Down-regulated molecule		
Interleukin-18	−167	−9.4
IGF-1	−29.6	−23.8
Cathepsin L	−10.5	−6.1
Cathepsin C	−5.1	−3.7
IL-6 receptor	−2.4	−1.9
Cathepsin B	−1.8	−2.0

Cells were incubated with RANK-L (60 ng/ml) in α-MEM for 5 days. Total RNA was isolated and utilized for real time PCR and Affymetrix chip analysis using u74Av2 mouse genome arrays. Results are shown as fold-increases (+) or decreases (−) compared with day 0. Real time PCR data were normalized to GAPDH and are representative of up to three separate experiments done in triplicate wells. Affymetrix data were obtained from duplicate RNA samples.

Amongst the cathepsins, only cathepsin K expression was dramatically increased, while cathepsins C, B, and L were reduced by 2- to 10-fold. In a separate set of experiments, there were no significant changes in the level of mRNA expression between 3 and 5 day treatment with RANK-L (Fig. 2). Only cathepsin K showed a marginally significant ($p = 0.07$) increase in expression at day 5 over day 3. These results not only help to confirm the role of cathepsin K in bone resorption [12], but also refute previous assumptions based on pharmacological inhibitor studies that have anecdotally implicated the cathepsins C, B, and L in matrix degradation [13–16]. Cathepsin K, when ablated genetically, resulted in osteopetrosis, indicating a significant role in resorption [17,18]. Interestingly, however, whereas there was no overt osteopetrosis evident in cathepsin L deficient mice, alterations in trabecular turnover, particularly in response to withdrawal of estrogen, were observed [19].

That IL-18 expression was reduced during late stages of osteoclast maturity (Table 1B) makes biological sense as the cytokine is known to inhibit osteoclast formation and bone resorption [20]. What was counterintuitive, however, is that receptor levels for IL-6 declined with osteoclast maturation, since IL-6 is osteoclastogenic [21] (Table 1B). Also, that IGF-1 mRNA was detected in the osteoclast lineage (Table 1B) raises the intriguing possibility that, in addition to regulating osteoblast differentiation, IGF-1 might modulate the formation and/or function of osteoclasts, since IGF-1 receptor is expressed in both pre-osteoclasts and mature osteoclasts [22,23].

We next examined differences in the expression of NFκB subunits p65, p50, c-*Rel*, IκBα, and IκBβ during osteoclast formation. Their expression at days 3 or 5 relative to their expression at day 0 was not significantly different (Fig. 2). Furthermore, comparisons between their relative expression at day 3 and day 5 yielded no significant differences, except for marginally significant trends ($0.1 > p > 0.05$) for IκBα and IκBβ, respectively. c-Rel showed a small decrease in expression at day 5 compared with day 3. Thus, RANK-L does not significantly alter the expression of the NFκB subunits.

Recent studies suggest that NFATc1 mediates osteoclast formation as a master molecule responsive to RANK-L [3,4,8]. Constitutively active NFATc1 regulates osteoclastogenesis in RAW264.7 cells [8] and antisense-transfected RAW264.7 cells fail to produce osteoclasts when exposed to RANK-L [3]. In our hands, constitutively active and dominant-negative NFATs stimulate and inhibit, respectively, RANK-L-induced osteoclast differentiation (Sun et al., unpublished).

NFATs are major substrates for the Ca^{2+}/calmodulin-activated heterodimeric phosphatase, calcineurin [5]. Inhibition of calcineurin by cyclosporin markedly reduces RANK-L-induced osteoclast differentiation [8]. Deletion of the α isoform of the catalytic subunit of calcineurin, calcineurin Aα, results in a marked reduction

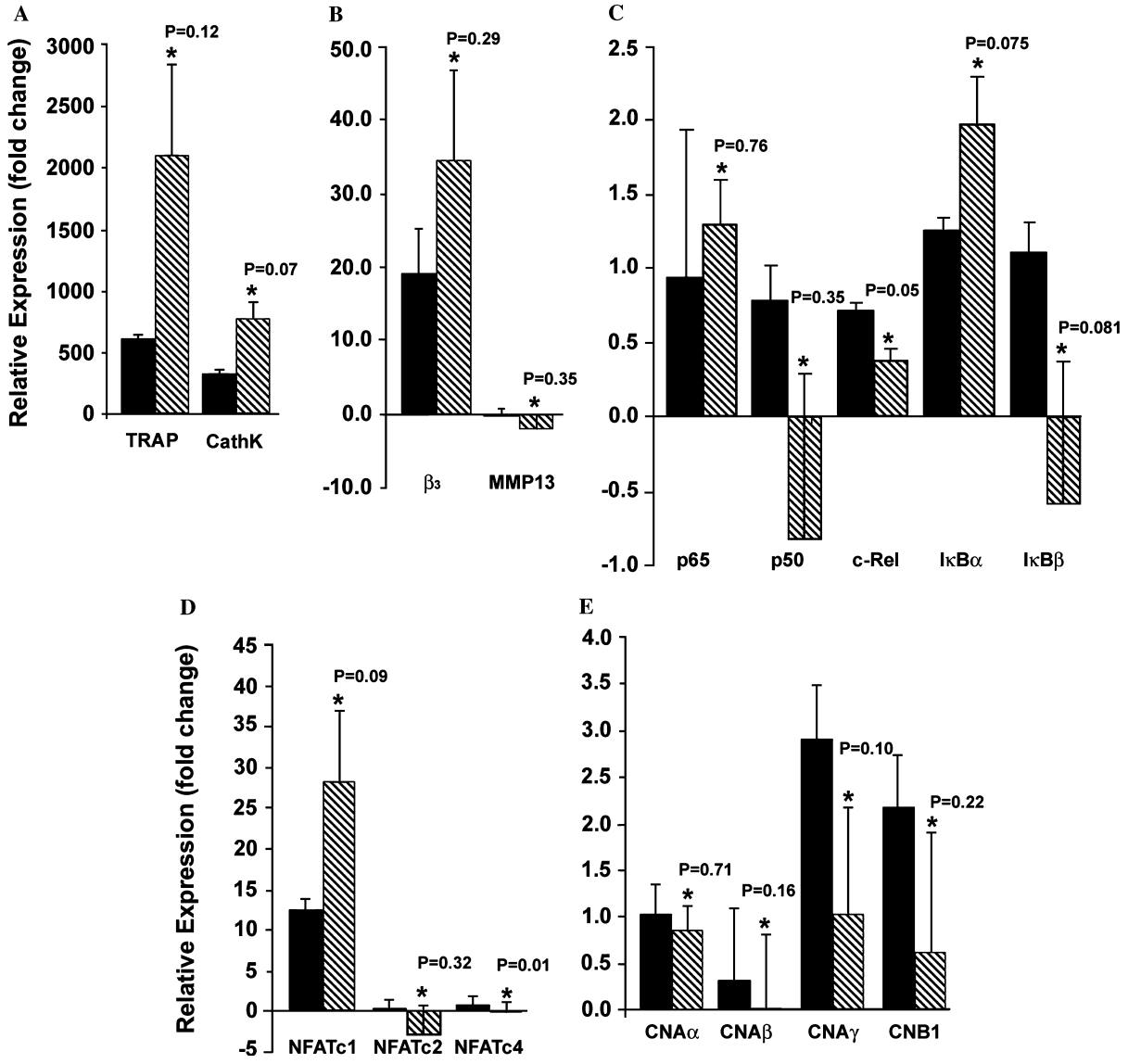

Fig. 2. Relative expression of various osteoclast genes: tartrate-resistant acid phosphatase (TRAP), cathepsin K (Cath K) (A), β_3 integrin, and metalloproteinase 13 (MMP13) (B), NFκB subunits (p65, p50, c-Rel, IκBα, and IκBβ) (C), NFAT isoforms (c1, c2, and c4) (D), and calcineurin subunit isoforms (Aα, Aβ, Aγ, and B1) (E). Measurements were made by real time PCR on RNA extracted from RAW264.7 cells exposed to RANK-L (60 ng/ml) for 3 days (bold bars) and 5 days (stippled bars). Means ± SEM are shown. Comparisons made between results at day 3 and day 5 are indicated by p values. Statistics: student's t test ($n = 3$).

in osteoclast formation. Consequently, calcineurin appears to be the Ca^{2+} sensor upstream of NFATc1 that dephosphorylates the transcription factor in response to changes in cytosolic Ca^{2+} and thus permits its nuclear localization. It is thus critical to examine the expression of the calcineurins and NFATs during osteoclast development.

RANK-L triggered a highly significant ~30-fold elevation in NFATc1 expression at day 5; this was not significantly different from that seen at day 3. Expression of the other NFAT isoforms at day 5 was not significantly different from that at day 0 or day 3 (<2-fold). Furthermore, the expression of the catalytic subunits of calcineurin (Aα, Aβ, or Aγ) did not change during osteoclastogenesis nor was the regulatory subunit affected. This indicates that RANK-L specifically regulates expression of NFATc1 in addition to the pathways leading to nuclear translocation and activation.

In summary, we have shown that reliable quantitative measurements of gene expression can be made by real time PCR that are broadly concordant with those obtained through Affymetrix chip assays. The study also confirms that a number of critical osteoclastogenic molecules, in particular those that are obligatory for osteoclastic bone resorption, such as cathepsin K, β_3, and

TRAP, are up-regulated during osteoclast formation from RAW264.7 cells in response to RANK-L. Finally, we also demonstrate that the *expression* of NFATc1 is RANK-L-regulated, whereas the expression of osteoclastogenic components of the NFκB system is not.

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