Expression of functional RANK on mature rat and human osteoclasts

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Abstract Although the important roles of RANK/RANKL in osteoclastogenesis have been established, their roles in the regulation of mature osteoclasts remain uncertain. Microisolation has been used to obtain pure populations of rat and human osteoclasts for RT-PCR analysis. RANK and calcitonin receptor mRNA was detected in all the samples whereas OPG and ALP mRNA was not present in any. RANKL mRNA was detected in two of eight rat and one of four human samples. Treatment of osteoclasts with soluble RANKL resulted in translocation of NF-κB to the nucleus and elevation of cytosolic and nuclear calcium levels. We have shown that RANK is highly expressed in mature osteoclasts and that its stimulation by RANKL results in activation of NF-κB and calcium signalling.

Key words: Osteoclast; Microisolation; RANK; RANKL; NF-κB; Intracellular calcium

1. Introduction

Receptor activator of NF-κB (RANK, TNFRSF11B) is a novel type I transmembrane receptor of the TNF receptor superfamily (TNFRSF) that was originally identified in a dendritic cell cDNA library [1]. It is ubiquitously expressed in human tissues, particularly skeletal muscle, thymus, liver, colon, intestine and adrenals [1] and also in trabecular bone, lung, brain and kidney [2]. Its ligand, RANKL, is involved in T-cell/dendritic cell interactions ([1] #167) and is identical to a type II membrane protein identified as TNF-related activation-induced cytokine (TRANCE) [3], osteoprotegerin ligand (OPGL) [4] and the long sought after 'osteoclast differentiation factor' (ODF) [5]. As recommended in a recent review, the term RANKL will be used to describe this novel protein [6]. Osteoprotegerin (OPG), a secreted protein that is also a member of the TNFRSF, binds to RANKL [7,8] thus preventing its interaction with RANK and the process of osteoclastogenesis. The availability of a soluble form of recombinant human RANKL, comprising the extracellular domain of the protein (sRANKL), has allowed the in vitro generation of large numbers of bone resorbing osteoclasts from hemopoietic precursors in the absence of stromal/osteoblast cells [4,5,9–11].

Whilst it has been shown that RANKL has an important role in osteoclast generation (reviewed by Takahashi et al. [12] and Suda et al. [6]) and that this is mediated by RANK present on osteoclast precursors [2], its role in the function of mature osteoclasts is less well defined. Nevertheless, some very useful data have been produced. Lacey et al. [4] treated impure populations of neonatal rat osteoclasts cultured on bone slices with sRANKL and showed stimulation of bone resorption that was not associated with increased osteoclast number. Fuller et al. [10] treated similar preparations of rat osteoclasts with sRANKL and found a rapid increase in pseudopodial motility and cell spreading, activation of bone resorption and increased survival. These investigators also found a high level of RANK mRNA in rat osteoclasts, using RT-PCR of microisolated cells (Professor T.J. Chambers, personal communication). Hsu et al. [13] used in situ hybridisation of embryonic and adult mouse bone to show RANK expression by osteoclasts, particularly those actively resorbing. Burgess et al. [14] have reported that sRANKL or anti-RANK antibodies (directed against the extracellular domain of RANK) rapidly induced actin ring formation in rat osteoclasts and that sRANKL increased the bone resorbing activity of these osteoclasts. Specific binding of FITC-labelled RANKL to osteoclasts was demonstrated. They also found that intravenous injection of sRANKL (0.05–0.5 mg/kg) raised calcium levels in mice within 1 h, the rapidity of the response suggesting an effect on activation of mature osteoclasts, rather than on osteoclast generation.

Genes associated with apoptotic mechanisms are activated by RANKL [15,16] and upstream signalling elements are required for this activity, particularly C-terminal binding regions that recognise TNF receptor-associated factors (TRAFs) [16]. Treatment of osteoclasts with sRANKL also activates c-Jun N-terminal kinase associated with nuclear factor-kappaB (NF-κB) activity [6,17]. As RANK has a large cytoplasmic domain that is not similar to that of other TNFRSF members it is possible that other functions or signalling pathways are yet to be ascribed to its activation.

The technique of microisolation is currently the only method of obtaining pure osteoclast preparations and we have previously used this in combination with RT-PCR to determine gene expression in osteoclasts [18,19]. In this study, we have employed the microisolation/RT-PCR technique to determine gene expression of RANK and related novel proteins

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Abbreviations: NF-κB, nuclear factor-kappaB; RANK, receptor activator of NF-κB; RANKL, RANK ligand; sRANKL, soluble RANKL; OPG, osteoprotegerin; TRANCE, TNF-related activation-induced cytokine; ODF, osteoclast differentiation factor; TNFRSF, tumour necrosis factor receptor superfamily; TRAF, TNF receptor-associated factor; CLSM, confocal laser scanning microscope; GCT, giant cell tumour (of bone); CTR, calcitonin receptor; VnR, vitronectin receptor; ALP, alkaline phosphatase; RT-PCR, reverse transcription-polymerase chain reaction; IL-1, interleukin-1; IL-6, interleukin-6; TNFα, tumour necrosis factor alpha; ECGF, epithelial cell growth factor

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in rat and human osteoclasts. In addition, we have used confocal laser scanning microscopy (CLSM) of single osteoclasts to determine whether RANKL treatment of osteoclasts results in activation of NF-κB and calcium signalling responses.

2. Materials and methods

2.1. Materials

Tissue culture medium was purchased from Sigma Chemical Co. (St. Louis, MO, USA) or TRACE (Melbourne, Australia) and FBS from CSL (Melbourne, Australia). Total cellular RNA was isolated with RNAzol B (Tel-Test, Inc., Friendswood, TX, USA). Diethyl pyrocarbonate (DEPC) was purchased from BDH (Poole, UK). A first strand complementary (cDNA) synthesis kit (Boehringer Mannheim, Germany) was used for RT reactions, and in PCR reactions, deoxynucleotide trisphosphates (dNTPs) were obtained from Promega (Madison, WI, USA). Reaction buffer, $MgCl_2$, Taq DNA polymerase and 100 bp DNA ladder were all supplied by Life Technologies (Gibco BRL, Gaithersburg, MD, USA). Vitronectin receptor (VnR) monoclonal antibody, 23c6, was generously provided by Prof. Michael Horton, University College, London, UK. Anti-mouse IgG-coated immunomagnetic beads were purchased from DYNAL Pty Ltd (Melbourne, Australia). Albumin (Haemopearl fatty acid-free) was purchased from ScimaR (Melbourne, Australia) and p65 antibody from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Fluo3 acetoxymethyl ester (Fluo3-AM) and other fluorescent probes were purchased from Molecular Probes (Eugene, OR, USA). Ionomycin, EDTA, and other agents for calcium regulation studies were purchased from Sigma (St. Louis, MO, USA).

2.2. Osteoclast purification and microisolation

Rat osteoclasts were disaggregated from the long bones of neonatal Wistar rats [20,21] and microisolated as previously described by Tong et al. [18]. In brief, osteoclasts where settled on plastic Petri dishes and washed vigorously to remove less adherent cells, leaving a sparse population of osteoclasts. The osteoclasts were detached from the surface using an EDTA buffer and collected with a micropipette.

Human GCT samples were obtained following informed consent from patients undergoing routine orthopedic surgery. Under aseptic conditions, the tumour tissue was teased apart and then passed through a 100 μm mesh to produce a suspension of cells. Osteoclasts from GCT were enriched by magnetic bead isolation (Dynal Dynabeads). VnR antibody 23C6 was used as previously described [22]. The beaded cells were isolated and washed using a magnetic concentrator to recover the cells. Typically, preparations were greater than 95% pure for VnR positive cells. This comprised mostly large multinucleated cells and to a lesser degree VnR positive mononuclear cells. The 23C6+ve multinucleate human GCT osteoclasts were microisolated as previously described [19].

2.3. RNA isolation and RT-PCR

The microisolated osteoclasts were lysed in RNAzol B solution and total RNA extracted according to the manufacturer's instructions. RNA was dissolved in 5 ml DEPC-treated H_2O and this total amount reverse transcribed to give a 20 μl aliquot of cDNA. A Perkin Elmer/Cetus DNA Thermal Cycler was used for RT-PCR. RT was performed in the presence of 5 mM $MgCl_2$, 1 mM deoxynucleotide mix, 3.2 μg random primers, 50 units RNase inhibitor and 20 units AMV reverse transcriptase. The final mixture was reacted at 25°C for 10 min, 42°C for 60 min and 95°C for 5 min to denature the enzyme. Sense and antisense primers were designed using the MacVector program and synthesised by Gibco BRL (Gaithersburg, MD, USA). Oligonucleotide primers for calcitonin receptor (CTR), RANK, RANKL, OPG and alkaline phosphatase (ALP) are described in Table 1.

Rat CTR primers enabled the identification of the two CTR isoforms that differ by inclusion or exclusion of a 37 amino acid insert. The resultant PCR fragments of 447 or 558 bp identify the C1a and C1b receptor, respectively [23]. Human CTR primers enabled the identification of the two CTR isoforms that differ by inclusion or exclusion of a 16 amino acid insert. The resultant PCR fragments were 364 and 412 bp [24]. Sequences and product sizes are defined in Table 1.

All PCR products were confirmed by restriction enzyme digest analysis and the rat RANK product was sequenced for further investigation. All the primer pairs spanned intron-exon splice sites allowing for the detection of mRNA only. One tenth of the cDNA was used in each PCR reaction. PCR was performed with cycles of denaturation at 95°C for 1 min, annealing at 55°C for 2 min and extension at 72°C for 1 min. The reaction mixture contained 40 pmol of each primer, 200 mM dNTPs, 2 ml of 10× reaction buffer, 1.0 mM $MgCl_2$ (except for human OPG, 0.75 mM; human CTR, 1.5 mM), 1 U Taq DNA polymerase, and sterile distilled water up to 20 μl. The mixture was overlaid with paraffin oil. Samples of cDNA from microisolated cells were amplified for 65 cycles while positive controls were amplified for 40 cycles only. PCR products were resolved on a 1.2% agarose gel and visualised using ethidium bromide. The sizes of the bands were confirmed by a 100 bp DNA ladder (Gibco BRL, Gaithersburg, MD, USA).

Positive controls used for gene expression in the rat were cDNA from brain (CTR), liver (RANK) and UMR106 cells (RANKL, OPG and ALP). For analysis of human samples, cDNA from whole GCT was used as this tissue expressed all genes of interest.

2.4. NF-κB activation in RANKL-treated osteoclasts: assay of p65 nuclear translocation

Osteoclasts were plated onto sterile 25 mm cover glasses as a meniscus and allowed to settle for 2 h, washed gently in medium and then incubated at 37°C in α-MEM containing 10% FBS for 4 h (rat) or 24–48 h (human) before commencing the experiments. Osteoclasts on cover glass were treated with sRANKL (30 ng/ml) or vehicle for 30 min. Cells were fixed in 4% paraformaldehyde, washed in PBS and permeabilised using 0.1% Triton X-100. Immunofluorescent staining was performed using a p65 polyclonal antibody (Santa Cruz, CA, USA) and secondary immunostaining with goat anti-rabbit IgG-FITC conjugate. The osteoclasts were viewed by CLSM, enabling reflection mode cell imaging as well as fluorescence output (BioRad 1024 system; Leica DMR-1B inverted microscope with epifluoresence; infinity-corrected 63× NA1.3 and 16× NA0.9 lenses with z-slice of 1.865 and 0.474 micron, respectively). Each population of cells was categorised by a blinded independent observer as positive or negative for p65 translocation to the nuclear membrane.

2.5. Analysis of intracellular calcium ion changes using CLSM

Intracellular Ca^{2+} changes were assessed using the Ca^{2+} indicator, Fluo3. Osteoclasts cultured on cover glass were mounted in an open perfusion chamber (Model PDMI-2 from Medical Systems Corporation, Greenvale, NY, USA) and maintained at 37°C using a bipolar temperature controller (Model TC-102). The cells were loaded for 20 min with 1 μM Fluo3-AM in M199 medium containing 0.1% Haemopearl fatty acid-free albumin (ScimaR, Australia). After loading, cells were washed with fresh medium and maintained at 37°C until processing. Time-course studies of the loading were performed to ensure adequate and steady-state loading. Both fluorescence and reflection mode images were collected so that shape change and cell movement could be assessed. Results were analysed by viewing cell movie sequences. Calcium ion changes were assessed using an NT-based MicroComputer Imaging Device (MCID) from Imaging Research (Berthold, Australia). Data from digitised montages of cell images were used to calculate changes in intensity of fluorescence after cell stimulation.

3. Results

3.1. Analysis of RANK, RANKL and OPG mRNA expression by RT-PCR in pure osteoclast preparations

Expression of RANK, RANKL and OPG mRNA was assessed in eight samples of microisolated rat osteoclasts and four samples of microisolated human osteoclasts. In all rat samples, CTR mRNA was present while ALP mRNA expression was not detected, suggesting the absence of significant osteoblast contamination (Fig. 1). All of the eight rat samples contained RANK mRNA, while RANKL mRNA was demonstrated in two of the eight (Fig. 1). OPG mRNA was not detected in any samples. Similar to the expression profile in rat osteoclasts, CTR and RANK mRNA were demonstrated

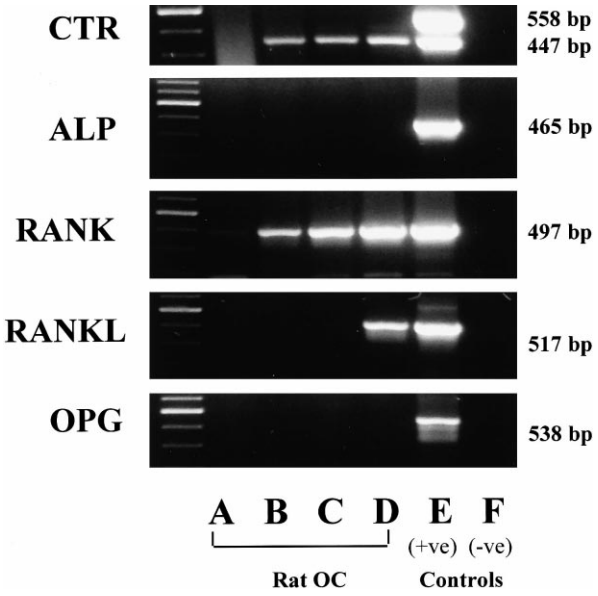

Fig. 1. RT-PCR analysis of microisolated rat osteoclasts. Pure rat osteoclasts were microisolated and total RNA was extracted. PCR analysis was performed using defined primers as described in Section 2. A to D represent four separate rat cDNA samples, representative of the eight tested. The positive control (sample E) is rat brain for CTR; rat liver for RANK; UMR106 cell line for ALP, RANKL and OPG. The negative control (sample F) has no added cDNA in each case. The CTR analysis of sample A was indeterminate in the experiment shown but was positive when the experiment was repeated.

in all four human osteoclast samples. RANKL mRNA was detected in only one human sample and OPG mRNA was not detected in any.

3.2. RANKL-induced NF-κB activation as assessed by p65 translocation to the nucleus

In six separate experiments (three rat and three human osteoclast preparations), p65 translocation to the nucleus was assessed from CLSM images. Three images from each of the control group and the sRANKL-treated group from a rat osteoclast experiment are presented (Fig. 2A). The fluorescent immunostaining of p65 in untreated cells was diffuse and distributed throughout the cytoplasm. As depicted in Fig. 2B, 14% of the control group as compared to 94% of the sRANKL-treated group were scored as positive for translocation. Perinuclear and intranuclear localisation of p65 occurred in the sRANKL-treated samples. ATP [25] and bradykinin [26], both positive regulators of NF-κB activity, were included as positive controls and both elicited strong p65 translocation to the nucleus in all samples (results not shown). ECGF was added as a negative control [27] and elicited no change in distribution of the fluorescence staining compared to control cells (results not shown).

3.3. RANKL-induced changes in intracellular ionic calcium levels

Intracellular Ca^{2+} levels were measured to determine whether RANKL action on osteoclasts and subsequent p65 translocation were associated with intracellular Ca^{2+} changes. The montage of the time sequence depicted in Fig. 3 was derived from a video sequence from the CLSM. Cell shape was monitored by reflection CLSM microscopy to enable simultaneous morphological assessment. No shape change occurred and calcium responses to EDTA and ionomycin were tested at the conclusion of each experiment. This ensured that changes in cell thickness had not affected fluorescence output and that membrane calcium channels were functional but not activated during cell preparation. The nuclear Ca^{2+} response occurred prior to the cytosolic response (Fig. 4) and at a faster rate. However, saturation occurred at approximately the same time for both. Calcium quantification is not possible in this model as the CLSM does not allow ratiometric assessment of calcium-sensitive dyes.

4. Discussion

In this study we have provided definitive evidence that RANK is expressed on mature rat and human osteoclasts and that treatment with RANKL results in activation of NF-κB and Ca^{2+} signalling in these cells. Others have shown that RANKL treatment of osteoclasts induces functional changes including increased bone resorption [4,10,14], increased cytoplasmic spreading and survival [10], and actin ring formation [14]. The finding that anti-RANK antibodies induce the same effects as RANKL is good evidence that the

Table 1
Primers used for RT-PCR

Gene sequence mRNA	Oligonucleotide sequence	Sequence based on Accession No.	Product (base pairs)
rat CTR (C1a and C1b isoforms)	5′-ACACCCTGACAGCAACCGAACCT-3′	L14617	447
	5′-GAACCCCCAGCCAAGTAAATAGTA-3′	L13040	558
human CTR isoforms	5′-GCAATGCTTTCACTCCTGAGAAAC-3′	HSU26554	364
	5′-CAGTAAACACAGCCACGACAATGAG-3′		412
rat and human RANK	5′-TTAAGCCAGTGCTTCACGGG-3′	AF018253	497
	5′-ACGTAGACCACGATGATGTCGC-3′		
rat RANKL	5′-ACGCAGATTTGCAGGACTCGAC-3′	AF019048	493
	5′-TTCGTGCTCCCTCCTTTCATC-3′		
human RANKL	5′-CAGCACATCAGAGCAGAGAAAGC-3′	AF019047	517
	5′-CCCCAAAGTATGTTGCATCCTG-3′		
rat OPG	5′-TGGCACACGAGTGATGAATGCG-3′	U94330	538
	5′-GCTGGAAAGTTTGCTCTTGCG-3′		
human OPG	5′-GTACGTCAAGCAGGAGTGCAATC-3′	U94332	472
	5′-TTCTTGTGAGCTGTGTTGCCG-3′		
rat and human ALP	5′-TCATGTTCCTGGGAGATGGTATG-3′	X16026, X14916, X16027, X14916	465
	5′-GCATTAGCTGATAGGCGATGTCC-3′		

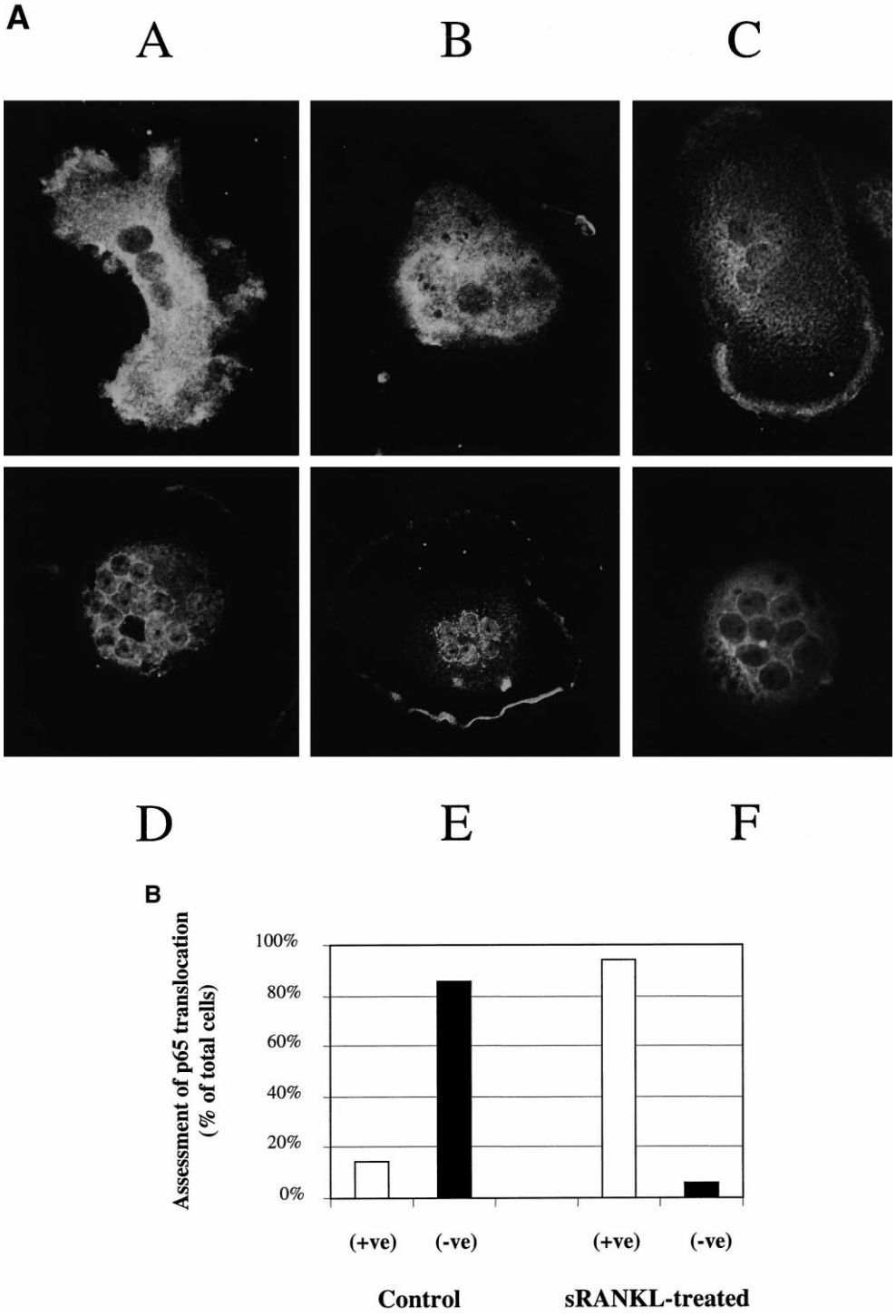

Fig. 2. A: RANKL-induced p65 translocation in rat osteoclasts. Neonatal rat osteoclasts were cultured on glass and treated with control medium or sRANKL (30 ng/ml) for 30 min. Cells were analysed for location of p65 by immunofluorescence using CSLM. Images A, B, C depict cells treated with control medium while images D, E, F illustrate p65 translocation to the nucleus, and typical perinuclear and intranuclear staining, after treatment with sRANKL. B: Assessment of sRANKL-induced p65 translocation in neonatal rat osteoclasts. Osteoclasts were assessed for the percentage of cells that showed positive p65 translocation. Cells were treated with either control medium or sRANKL (30 ng/ml). Results are representatives of three independent experiments.

effects of RANKL on osteoclasts are mediated by RANK, as shown in osteoclast precursors [2,13].

RANKL mRNA expression was detected by RT-PCR in 25% of the specimens examined and, therefore, RANKL protein production by osteoclasts cannot be excluded. RANKL mRNA expression in osteoclasts may be uniformly low and near the threshold of detection by RT-PCR or, alternatively, may be heterogenous and detection in some of our samples was due to a higher representation of putative RANKL-producing osteoclasts in these samples. Another possibility is that, despite the absence of detectable ALP mRNA signal, there was minor stromal/osteoblast contamination in the sam-

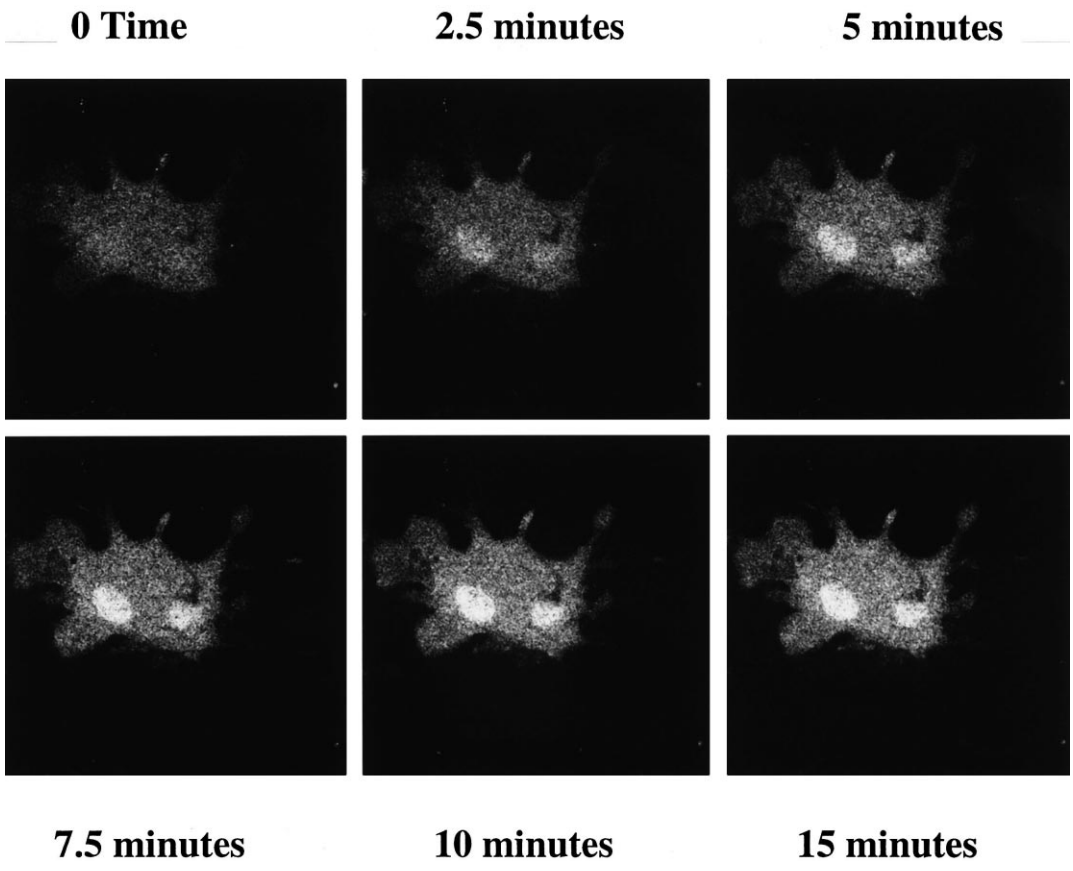

Fig. 3. Calcium ion changes in rat osteoclast induced by sRANKL. Neonatal rat osteoclasts, cultured on glass, were loaded with Fluo3-AM. Cells were equilibrated in an environmental chamber prior to addition of sRANKL (40 ng/ml) and the intracellular changes in fluorescence measured to determine temporal changes in Ca^{2+}. Images taken from a 36 image movie sequence are presented as a montage with time of stimulation indicated in min.

ples. If RANKL is indeed expressed in osteoclasts, or a subpopulation of osteoclasts, its functional role in this context remains to be defined. A paracrine/autocrine role of RANKL in osteoclast recruitment, survival or activation is possible. In contrast, OPG mRNA was not detected in any of the 12 samples, suggesting that OPG expression is not a feature of the osteoclast phenotype.

No previous studies have shown definitive association of RANK signalling with RANKL stimulation in live osteoclasts. The study of intracellular signalling mechanisms in osteoclasts using standard techniques has been limited by difficulties obtaining sufficient quantities of purified osteoclasts. To overcome this problem we have used CLSM to examine sRANKL-induced cellular responses, namely p65 translocation and intracellular Ca^{2+} changes, in individual osteoclasts. In the current study, immunofluorescent labelling showed that p65 translocation coincides with intracellular, and particularly intranuclear, mobilisation of Ca^{2+}. Analysis of the live cell Ca^{2+} assay with both reflection and fluorescence imaging on the CLSM confirmed that the cells remained viable and did not undergo dramatic shape change during the incubation period.

A number of cytokines can activate NF-κB and diverse genes can be regulated through the NF-κB signalling pathway. This topic has been reviewed [28–30]. Osteopetrosis due to deficient osteoclast formation occurs in p50/p52 knockout mice [32,33] and as described by Suda et al. [6] RANKL induction of p65 translocation is involved in osteoclast formation. Associations of NF-κB activity, bone cell function, and bone pathology have been postulated [31,32]. IL-1, IL-6 and TNFα activate NF-κB and lack of negative modulation of these cytokines may be related to the pathology of meta-

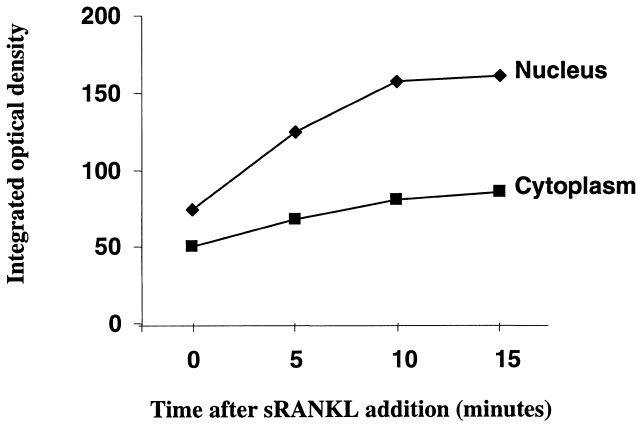

Fig. 4. Time-course of sRANKL-induced intracellular calcium ion changes in rat neonatal osteoclasts. Data from CLSM images in Fig. 3 were converted for analysis. Fluorescence intensity was quantified using an MCID program and data from 0, 5, 10 and 15 min plotted as integrated optical density per unit area in arbitrary units. Similar results were obtained in each of three experiments.

bolic bone disease. Pertinent to bone physiology, estrogen has been proposed as a modulator of NF-κB activity, the corollary being that lack of estrogen predisposes individuals to osteoporosis [31].

In the present study, the sRANKL-induced increase in intracellular Ca^{2+} in osteoclasts occurred within the time-frame of the translocation of NF-κB-p65 to the nuclear membrane. Elevated Ca^{2+} levels are required for nuclear localisation of transcriptional regulators in other systems [34–36] and a number of transcriptional regulators require the action of Ca^{2+} or even a calcium stress [37] for activity. Some transcription factors of the TNFRSF require changes in the cytosol [38,39] or in the nucleus for gene regulation [40] and both cytosolic and nuclear Ca^{2+} changes have been reported for cytokines and agonists that activate osteoclasts. IL-1 and ATP, both activators of NF-κB, can induce intracellular Ca^{2+} signalling in osteoclasts relevant to receptor activity [41–43]. Thus, our demonstration that nuclear Ca^{2+} changes are associated with sRANKL activation of osteoclasts adds another signalling pathway to those already identified that may be required for transcriptional events associated with RANK signalling.

In conclusion, we have shown that RANK mRNA is expressed in mature rat and human osteoclasts and that sRANKL activates signalling pathways associated with the RANK receptor. Our demonstration of sRANKL induction of nuclear and cytosolic Ca^{2+} changes may be relevant in osteoclast activation. Further analysis of sRANKL effects on pure populations of osteoclasts may identify other pathways involved with osteoclast function.

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