Expression of and Role for Ovarian Cancer G-protein-coupled Receptor 1 (OGR1) during Osteoclastogenesis*

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Osteoclasts differentiate from hematopoietic mononuclear precursor cells under the control of both colony stimulating factor-1 (CSF-1, or M-CSF) and receptor activator of NF-κB ligand (RANKL, or TRANCE, TNFSF11) to carry out bone resorption. Using high density gene microarrays, we followed gene expression changes in long bone RNA when CSF-1 injections were used to restore osteoclast populations in the CSF-1-null toothless (csf1tl/csft1tl) osteopetrotic rat. We found that ovarian cancer G-protein-coupled receptor 1 (OGR1, or GPR68) was strongly up-regulated, rising >6-fold in vivo after 2 days of CSF-1 treatments. OGR1 is a dual membrane receptor for both protons (extracellular pH) and lysolipids. Strong induction of OGR1 mRNA was also observed by microarray, real-time RT-PCR, and immunoblotting when mouse bone marrow mononuclear cells and RAW 264.7 pre-osteoclast-like cells were treated with RANKL to induce osteoclast differentiation. Anti-OGR1 immunofluorescence showed intense labeling of RANKL-treated RAW cells. The time course of OGR1 mRNA expression suggests that OGR1 induction is early but not immediate, peaking 2 days after inducing osteoclast differentiation both in vivo and in vitro. Specific inhibition of OGR1 by anti-OGR1 antibody and by small inhibitory RNA inhibited RANKL-induced differentiation of both mouse bone marrow mononuclear cells and RAW cells in vitro, as evidenced by a decrease in tartrate-resistant acid phosphatase-positive osteoclasts. Taken together, these data indicate that OGR1 is expressed early during osteoclastogenesis both in vivo and in vitro and plays a role in osteoclast differentiation.

The catabolic removal of bone during skeletal formation and remodeling requires the specialized activity of multinucleated osteoclasts. Osteoclasts differentiate by fusion of hematopoietic mononuclear precursors in response to systemic and local signals, in particular colony-stimulating factor-1 (CSF-1,2 or M-CSF) and the tumor necrosis factor family member receptor activator of NF-κB ligand (RANKL, or TRANCE, TNFSF11) (1). Excessive osteoclast activity systemically leads to osteopenia such as osteoporosis, whereas local hyperactivity can lead to osteolysis as seen in tumor metastases to bone or in prosthetic loosening. Hypoactivity of osteoclasts can lead to sclerotic bone disorders, for example in genetic conditions such as osteopetrosis.

Osteoclast differentiation is a complex process that requires the coordinated action of many gene products, including not only extrinsic factors such as CSF-1 and RANKL, which are supplied by osteoblasts locally in bone tissue, but also intrinsic factors required for osteoclast function. Mononuclear precursors must migrate to sites where resorption is needed, fuse to form multinucleated pre-osteoclasts, and attach firmly to bone. They develop highly specialized cellular structures, including an actin ring that forms a tight seal with the bone surface, and a highly convoluted plasma membrane domain called the ruffled border, which is the site of extremely active transport of ions, proteins, and membrane-bounded vesicles. The resorption lacuna underlies the osteoclast and is the site of bone removal. Mineral is dissolved by the low pH achieved by ATPase proton pumps, and the proteinaceous extracellular matrix is degraded by proteases. The digested bone is taken up by the osteoclast by endocytosis and exits the opposite side of the cell via transcytosis (2). Differentiation of osteoclasts from precursors takes roughly 4 days in vivo (3).

Many of the genes needed for this complex process have been identified through studies of osteopetrotic animal models and human osteopetrotic patients (1, 4). One such model is the toothless (tl) rat, in which a frameshift mutation in the csf1 gene results in severe osteopetrosis in homozygous mutants due to a nearly complete lack of osteoclasts (5, 6). Injections of recombinant CSF-1 restore osteoclast populations within several days in this animal model (3). We studied gene expression changes by high density microarray in the long bones of CSF-1-treated csf1tl/csft1tl rats over a time course. We previously reported on chemokine and chemokine receptor expression in this model.

2 The abbreviations used are: CSF-1, colony-stimulating factor 1; OGR1, ovarian cancer G-protein-coupled receptor 1; RT-PCR, reverse transcription PCR; BMC, bone marrow mononuclear cells; RANKL, receptor activator of nuclear factor κB ligand; siRNA, small inhibitory RNA; TRAP, tartrate-resistant acid phosphatase; SPC, sphingophosphorylcholine; NFATc1, nuclear factor of activated T-cells c1; PBS, phosphate-buffered saline; PBSTA, PBS with 1% bovine serum albumin; tl, toothless; CCLS, CC chemokine ligand 5.

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Experimental Procedures

Animals and Tissue Samples—Procedures involving animals were carried out under Institutional Animal Care and Use Committee-approved protocols at the University of Massachusetts Medical School. Restoration of osteoclasts in tl/tl rats was done by CSF-1 injections as described (3). Briefly, 3-week-old csf1 tl/csftl rats were obtained from the inbred colony maintained under specific pathogen-free conditions. Mutants were genotyped by neonatal x-ray and by PCR (6). Beginning at 21 days after birth, animals received daily injections of 10^6 units of recombinant human CSF-1 (generously provided by Chiron Corp., Emeryville, CA). At appropriate time points, tibiae and femora were harvested. For RNA extraction, bones were carefully dissected to remove extraneous connective tissue and muscle; joint cartilage and epiphyseal growth plates were also removed, leaving the metaphysis and diaphysis. Bones were split longitudinally and flushed with cold PBS to remove as much marrow as possible, although in untreated and 2-day-treated mutants, no discernible marrow spaces had formed. Dissected bones were flash frozen in liquid nitrogen for later RNA extraction (3).

Cell Culture—Mouse mononuclear bone marrow cells (BMC) were isolated and cultured as described (3, 14). Briefly, marrow cell suspensions were centrifugally fractionated on Histopaque 1077 (Sigma) and plated in low CSF-1 (10 ng/ml, human recombinant CSF-1; Chiron) in α-minimum essential medium (Invitrogen) supplemented with 10% fetal bovine serum (HyClone, Logan, UT). After allowing stromal cells to adhere overnight, non-attached cells were collected and plated in high CSF-1 (75 ng/ml). After 3 days, the medium was removed along with non-attached cells, and new medium containing CSF-1 (75 ng/ml) and RANKL (100 ng/ml; Peprotech, Rocky Hill, NJ) was added. Differentiation into osteoclasts was scored 3 days later by counting multinucleated (three or more nuclei) TRAP-positive cells. TRAP staining was done as described (3). RAW 264.7 pre-osteoclast-like cells were obtained from ATCC and plated at a density of 0.5–0.6 × 10^4 cells/well on 96-well polystyrene plates (Corning, Corning, NY) grown in the same medium as BMC but lacking CSF-1. Differentiation was induced by adding RANKL (100 ng/ml) and allowing 2–4 days to differentiate. In some experiments, osteoclast differentiation was modulated by addition of rabbit polyclonal anti-OGR1 antibody. Two different antibodies were tested, with essentially identical results (affinity purified, LifeSpan Biosciences, Seattle, WA; ammonium sulfate fraction, ExAlpha Biologicals, Watertown, MA) at concentrations of 1 and 2 μg/ml at the same time as RANKL was added. Negative controls consisted of an irrelevant antibody, rabbit polyclonal anti-CCL5 (affinity purified; Peprotech), and an ammonium sulfate fraction of non-immune rabbit serum (ExAlpha). At least six replicate wells were counted in at least two independent experiments by two different people for each experimental condition. In these experiments, greater sensitivity and reproducibility of the antibody effect was obtained with a lower concentration of RANKL, 33 ng/ml.

In the present study, we report on expression of ovarian cancer G-protein-coupled receptor-1 (OGR1) (GenBank accession XP_234483), which underwent rapid response to CSF-1 injections in the tl rat.

OGR1 is a member of a family of four known G-protein-coupled receptors with wide expression that selectively bind both protons and bioactive lipids and act through G, and Gq proteins (7, 8). OGR1 was initially cloned from an ovarian cancer cell line (9), and its ability to bind the bioactive lipid sphingophosphorylcholine (SPC) was shown in transfection studies (10). Those authors showed that SPC binding to OGR1 resulted in increased intracellular Ca^2+ and p42/44 mitogen-activated protein kinase activity and the internalization of OGR1. Although that report was recently withdrawn over concerns of falsified data by one of the authors (11), others have gone on to show that SPC is a legitimate ligand (12). A proton binding, pH-sensing activity of OGR1 was subsequently demonstrated and shown to depend on several His residues that reside in the extracellular domains of this seven-pass transmembrane protein (7). OGR1 expression was identified in human osteosarcoma cells and osteoblast precursor cells as well as in rat osteoblasts and osteocytes in situ, leading those authors to propose a role for the receptor in pH homeostasis via skeletal metabolism.

Despite these efforts, a clear biological function of OGR1 has yet to emerge. Recent work found that SPC antagonized the pH response of stably transfected Chinese hamster ovary cells and, further, that low extracellular pH caused an increase not only in phospholipase C (measured as inositol phosphate production) but also in cAMP, presumably via adenyly cyclase acting through G, proteins (12). In a recent report (13), OGR1 was found to be up-regulated in RAW264.7 pre-osteoclast-like cells in response to RANKL. That report also showed that reduction of extracellular pH in osteoclasts resulted in nuclear translocation of NFATc1, a downstream mediator of RANKL differentiation effects, although no specific physiological role for OGR1 in that process was demonstrated. Thus, although much remains to be learned about the actions of OGR1 in vivo, there is very suggestive evidence that it may mediate, at least in part, the role of the skeleton in pH homeostasis.

In our screens of gene expression changes during osteoclastogenesis in long bones of CSF-1-treated toothless osteopetrotic rats, we noted that OGR1 mRNA increased sharply during the early phase of osteoclast differentiation, i.e. during the first 48 h. This is prior to the appearance of significant numbers of mature, tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts in long bones and coincides with early events in osteoclastogenesis such as the production of the chemokine CCL9 by committed mononuclear precursors (3). We have investigated the expression of OGR1 during osteoclast differentiation, and we report that interference with OGR1 by antibody or siRNA causes significant decreases in osteoclast differentiation. This represents the first demonstration of biological activity for OGR1 in such a complex cellular process.
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**Immunofluorescence**—RAW 274.7 cells were grown on coverslips in 24-well plates with or without RANKL; after 60 h, they were fixed in 4% paraformaldehyde in phosphate-buffered saline for 10 min followed by two rinses in PBS. Cells were permeabilized with 0.1% Triton X-100 (Sigma) for 2 min and then rinsed twice with PBS followed by a 10-min incubation in PBS with 1% bovine serum albumin (PBSA; Sigma). Rabbit polyclonal anti-OGR1 (Lifespan) was diluted 1:20 in PBSA and incubated for 2 h at 37 °C with fixed cells (or with just PBSA for negative controls). Following four rinses in PBSA, coverslips were incubated with secondary antibody, donkey anti-rabbit IgG conjugated to Alexa 488 fluorochrome (Molecular Probes, Eugene, OR) for 1 h at 37 °C. Coverslips were rinsed twice with PBSA, once with PBS + 5 μg/ml 4',6-diamidino-2-phenylindole + 0.1% Triton X-100, and once with PBS. Cells were mounted on Antifade Gold (Molecular Probes). Images were obtained with a Zeiss Axio phot with a ×100 objective, equipped with a Zeiss AxioCam HR camera and Zeiss Axiovision software. Exposures times were 400 μs for the 4',6-diamidino-2-phenylindole wavelength and 800 μs for Alexa 488.

**Immunoblots**—CytoBuster reagent (Novagen, La Jolla, CA) was used to extract proteins from RAW 264.7 and BMC with and without RANKL treatment for 48 h. Protein was measured by Bradford assay (Bio-Rad), and equal amounts per lane were separated on 10% SDS-PAGE minigels and blotted onto polyvinylidene difluoride membrane (Immobilon P; Millipore, Billerica, MA) using a semidry transfer device (Owl Scientific, Cambridge, MA). After blocking in 5% nonfat dry milk in Tris-buffered saline, rabbit polyclonal anti-OGR1 antibody (X1600P; ExAlpha) diluted 1:100 in Tris-buffered saline containing 0.1% Tween 20 and 1% milk was added, and the blot was incubated for 90 min at room temperature. Following washes in Tris-buffered saline, goat anti-rabbit IgG alkaline phosphatase conjugate (Bio-Rad) was added, followed by more Tris-buffered saline washes. The blots were developed with Attoglow chemiluminescent substrate (Michigan Diagnostics, Troy, MI).

**RNA Extraction and Microarray Analysis**—Each time point was measured in each of four animals using RNA from cleaned limb bones (one tibia and one femur) as described (3). Briefly, frozen bones were pulverized in a Bessler mortar and pestle cooled with dry ice, and RNA was extracted from the bone powder using TRIzol (Invitrogen). After extraction, RNA was processed for microarray analysis according to Affymetrix instructions. RNA was cleaned using RNeasy columns (Qiagen, Valencia, CA), reverse transcribed (Superscript; Invitrogen), and transcribed in vitro to make cRNA with biotin incorporated (ENZO RNA in vitro transcript kit, Farmingdale, NY). The cRNA was then subjected to limited alkaline hydrolysis before hybridization with the DNA microarrays. High density microarray analysis was done using the RAE230 rat chip set (Affymetrix) according to the manufacturer’s instructions. This set represents some 30,200 different cDNA sequences on two chips. For each chipset, we tested RNA from a single animal. Chip image files were obtained through Affymetrix GeneChip software (MAS5). Subsequently D-Chip (www.biostat.harvard.edu/complab/dchip/) analysis was performed to analyze differentially expressed genes, and further analyses were done using Excel (Microsoft, Redmond, WA). Expression levels are means for four individual animals, given in relative units. Microarray screens were also done on differentiating mouse BMC and RAW 264.7 cells following essentially the same procedures but using the mouse chipset MOE 430. In those screens, only a single chipset was used for each experimental condition.

**siRNA Transfection Experiments**—Proliferating RAW 264.7 cells were plated as above. The next day, cells were transfected with siRNA using siIMPORTER (Upstate, Lake Placid, NY) according to the manufacturer’s specifications and treated with 100 ng/ml RANKL. The control (siRNA Silencer Negative Control 1 siRNA) was obtained from Ambion (Austin, TX). The two siRNAs targeted for OGR1 were obtained from Invitrogen: Gpr68-MSS215573, 5'-UAACAUUCCAGGUUCCGCCC-UGG-3' (‘si 73”), and Gpr68-MSS215574, 5'-UAGAAG-GAAAGGCAAGGAGAGAAGG-3' (‘si 74”). Forty-eight hours after transfection, total RNA was extracted from some wells and analyzed for OGR1 mRNA using real-time RT-PCR. Seventy-two hours post-transfection, other wells were stained for TRAP activity (Sigma) and assayed microscopically for the total number of TRAP-positive cells/well with three or more nuclei. The counts were done by two individuals, and the results were pooled. Each experimental condition was tested in six replicate wells for each siRNA, and experiments were carried out three separate times. Results for each well were expressed as “percent siRNA control,” and the data from the three experiments were pooled.

**Real-time PCR**—RNA was isolated and reverse transcribed, and real-time RT-PCR was carried out as described (14). To assess any effects of transfection, etc., on cell viability or vigor, we compared total RNA yield/well. Cell counts are problematic due to the fact that cells were fusing, so counts do not reflect the health of the culture. In all experimental conditions compared in this report, the total RNA yield/well was ±10% control values. Total RNA was extracted from cells or tissue using TRizol (Invitrogen) according to the manufacturer’s specifications and reverse transcribed into cDNA using the Superscript First Strand Synthesis System (Invitrogen). The cDNA was used as template for real-time PCR analysis. Reactions were set up in microcapillary tubes using 1 μl of cDNA with 9 μl of a LightCycler FastStart DNA Master SYBR Green I mix (Roche Diagnostics) to which gene-specific upstream and downstream PCR primers had been added. The final concentrations of the reaction components were 1.0 μM of each primer. Analysis was performed in a LightCycler instrument (Roche Applied Diagnostics) according to the manufacturer’s instructions. The primers for OGR1, obtained from Integrated DNA Technologies, Inc. (Corvalle, IA), were 5’-AAAGGCAATCTAGTGTGGTATGGG-3’ (forward) and 5’-AGGATCTAGGATCATCAC-TTGTTGTA-3’ (reverse). Values were normalized to glyceraldehyde-3-phosphate dehydrogenase. Primers for glyceraldehyde-3-phosphate dehydrogenase were 5’-GACCCTCTCATTGACCTCAAC (forward) and 5’-TACTCAGCACC-GCCTCACC-3’ (reverse).

**Statistical Tests**—Data were analyzed using statistical functions in Microsoft Excel and are shown as mean ± 1 S.D. F-tests
were done to determine whether variances were equal using a $p < 0.05$ cutoff for significance. Following that, $t$-tests were performed for significance of differences in sample means, again with a cutoff of $p < 0.05$.

RESULTS

OGR1 mRNA in $t/l$ Osteopetrotic Rat Long Bones following CSF-1 Treatments—Toothless ($csf1^{tl}/csf1^{tl}$) osteopetrotic rats lack osteoclasts due to an inactivating frameshift mutation in the $csf1$ gene (5, 6). Injections of CSF-1 restore osteoclast populations, with a few TRAP-positive cells first appearing in long bones 2 days after initiation of CSF-1 treatments and reaching a peak at 4 days (3). We analyzed RNA isolated from tibiae and femora of CSF-1-treated $t/l$ rats by high density microarray analysis to identify genes differentially regulated during this rapid burst of osteoclast differentiation in vivo. One of the genes that showed a striking and rapid response to CSF-1 was OGR1. On the rat arrays, OGR1 was represented by an expressed sequence tag from the 3′-untranslated region of the mRNA, GenBank™ accession BM389005. As shown in Fig. 1, OGR1 mRNA in long bone RNA increased rapidly, reaching a peak nearly 7-fold higher than in untreated $csf1^{tl}/csf1^{tl}$ mutants. Starting at a baseline level of 256 ± 20 relative units (mean ± S.D.), it rose to 1717 ± 748 relative units following 2 days of CSF-1 injections. This dropped slightly to 1273 ± 410 relative units on day 4 and to 782 ± 197 relative units on day 6. All these
differences were highly significant compared with the baseline value \((p < 0.01)\) and were verified by real-time RT-PCR (not shown). We showed previously that osteoclast counts in long bones followed a later time course, peaking on day 4 (3). Thus, the peak in OGR1 precedes the peak in osteoclast counts by 2 days.

**OGR1 Expression during Osteoclast Differentiation In Vitro**—Because long bones are complex tissues, we sought to identify the cellular source of the large increase in OGR1 mRNA. We found only low levels of OGR1 mRNA in cultured calvarial osteoblasts (data not shown). The timing of the rise paralleled that seen for other molecules that act early in osteoclast differentiation, such as the chemokine CCL9 and its receptor CCR1 (3). We therefore investigated increases seen in vivo in differentiating cultures of osteoclasts, both in mouse primary bone marrow mononuclear cells and in the pre-osteoclast-like mouse cell line RAW 264.7. Microarray analysis was performed on RNA isolated from differentiating cultures after 2 and 4 days of exposure to RANKL, and the results are shown in Fig. 2, A and B. In this screen a single chip set was used per condition, so no statistics are given. The mRNA increases were confirmed at the protein level by Western blots (Fig. 2D) of BMC and RAW cell protein extracts without (−) and with (+) 48 h of RANKL exposure. The specificity of the antibody is shown in the left panel of Fig. 2C, which gave a single band in all extracts tested (RAW cells shown); the increases in OGR1 protein in both cell types is shown in the right panel. RANKL induced a 6-fold rise in OGR1 mRNA in BMC by 2 days and dropped to roughly 5-fold by 4 days of RANKL treatment. In RAW 264.7 cells, OGR1 mRNA increased ∼2-fold in response to RANKL and remained elevated through day 4. Thus, the rise in OGR1 mRNA seen in total long bone RNA could be reproduced using isolated osteoclast lineage cells. Subsequent real-time RT-PCR confirmed the results for RAW 264.7 cells (see time course in Fig. 3) and for BMC (data not shown).

A next question was whether the rise in OGR1 mRNA was an immediate response to RANKL signaling or required other downstream events before its expression increased. We therefore measured OGR1 mRNA in RAW 264.7 cells by real-time RT-PCR in response to RANKL over a 96-h time course, and the results are shown in Fig. 3. The increase in OGR1 mRNA at 2 h was statistically significant \((p < 0.05)\), but slight. At 24 h, it was roughly 2-fold above baseline \((p < 0.005)\), at 48 h, ∼4-fold above baseline \((p < 0.01)\), and at 96 h, ∼2.5-fold greater than baseline \((p < 0.05)\). This is consistent with OGR1 not being an immediate target of RANKL signaling.

**OGR1 Protein in Differentiating RAW Cells**—We performed indirect immunofluorescence on differentiating RAW 264.7 cells to examine the induction and distribution of OGR1 protein; the results are shown in Fig. 4. Cells incubated with RANKL for 60 h were strongly labeled by anti-OGR1 antibody, whereas in cells not treated with RANKL the signal was consistently weaker (compare panels C and D), consistent with the Western blots. In Fig. 4D, a large, multinucleated osteoclast can be seen with high OGR1 in the cytoplasm and in the lamellipodial...

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**FIGURE 3.** Time course of OGR1 induction by RANKL. RAW 264.7 cells were exposed to RANKL for the number of hours shown, and OGR1 mRNA levels were measured by real-time RT-PCR normalized to glyceraldehyde-3-phosphate dehydrogenase. Differences were statistically significant at 2, 24, 48, and 96 h \((p < 0.05, *; p < 0.005, **; p < 0.01, ***; p < 0.05, *\); respectively). Mean ± 1 S.D. is shown, \(n = 3\).

**FIGURE 4.** OGR1 immunofluorescence in RAW 264.7 cells. RAW cells incubated with (B, D) or without (A, C) RANKL for 60 h. Negative controls had no primary antibody (A, B). All panels had the same exposures for 4',6-diamidino-2-phenylindole (DNA, blue) and for Alexa 488-conjugated secondary antibody (OGR1, green). Bar in panel A indicates 24 μm in all panels. Note the intense expression of OGR1 in the RANKL-treated cells (D) compared with control cells incubated without RANKL (C). 6–8 nuclei can be seen in the large osteoclast in panel D, which is stained intensely for OGR1 protein. Mononuclear cells also showed consistent, high OGR1 label (filled arrows in panel D), and lamellopodia at the cell periphery also contained OGR1 (open arrows).
extensions (open arrows). Negative controls without anti-OGR1 gave no signal whether RANKL was added or not (Fig. 4A and B). Also, we routinely observed mononuclear cells in RANKL-treated wells with very high levels of OGR1 (Fig. 4D, solid arrows), consistent with OGR1 being an early event not dependent upon mononuclear cell fusion or later maturation events.

A Role for OGR1 in Osteoclast Differentiation—The peak in OGR1 mRNA in long bones preceded that for the osteoclast marker TRAP mRNA and the appearance of mature, TRAP-positive osteoclasts by a full 2 days (3). We also saw strong expression in mononuclear RAW cells after RANKL treatment (Fig. 4). This suggested a role in early steps of osteoclast differentiation and not simply in late stage function required for actual bone resorption. We therefore undertook inhibition studies in differentiating cultures of murine BMC and RAW 264.7 cells. When affinity-purified anti-OGR1 antibody (LifeSpan) was added to differentiating cultures, osteoclast differentiation was significantly inhibited in a dose-dependent manner (Fig. 5). Both cell types had significant reductions in osteoclasts/well at both antibody concentrations tested. BMC averaged 22.7 ± 5.9 osteoclasts/well without anti-OGR1, whereas with 1 μg/ml antibody there were 11.7 ± 3.9 (p < 0.005) osteoclasts/well, and with 2 μg/ml antibody there were 8.0 ± 2.8 osteoclasts/well (p < 0.001). The dose response, i.e. the difference between 1 and 2 μg/ml antibody, was also significant (p < 0.05). RAW cell differentiation was similarly inhibited by anti-OGR1 antibody. With no antibody, there was an average of 20.4 ± 4.5 osteoclasts/well; with 1 μg/ml of antibody there were 10.4 osteoclasts/well (p < 0.001), and with 2 μg/ml, there were 7.8 ± 1.8 osteoclasts/well (p < 0.001). Again, the difference between the doses was significant (p < 0.005). Essentially identical results were obtained with an independent rabbit polyclonal anti-OGR1 antibody (ExAlpha) (not shown). Negative control experiments used an irrelevant affinity-purified antibody, anti-CCL5, and a non-immune rabbit ammonium sulfate serum fraction. Neither had any significant effect on osteoclast differentiation (data not shown).

Further investigation of OGR1 in osteoclast differentiation was carried out using siRNA to reduce the mRNA level and to measure the effect on osteoclast differentiation in RAW cells. Two siRNA oligonucleotides were tested for their effect in down-regulating OGR1 mRNA and the impact of this on osteoclast differentiation. The results are shown in Fig. 6. Both siRNA oligonucleotides had significant effects in reducing OGR1 mRNA (Fig. 6A). The oligonucleotide designated si73 caused a reduction to 41 ± 17.7% and si74 caused a reduction to 51.2 ± 16.4% compared with cells transfected with the negative control siRNA (p < 0.005 for both). They also produced highly significant reductions in osteoclast counts/well (Fig. 5B). Oligo si73 caused a reduction to 55.7 ± 10.6% of controls (p < 0.0001), and si74 caused a reduction to 55.7 ± 12.9% (p < 0.0001). To rule out possible effects on cell survival or overall viability as a cause of decreased osteoclast counts, we measured the total RNA yield from all wells tested and they did not differ significantly between experimental groups (data not shown).

DISCUSSION

It is well established by many lines of evidence that pH regulation of intracellular and extracellular compartments is essential to osteoclast activity. For example, the loss of acidification of the resorption lacuna due to mutations either in the 6i subunit of the vacuolar ATPase proton pump, in the chloride channel CLC7, or in carbonic anhydrase results in catastrophic failure of osteoclast activity and severe osteopetrosis in human patients and animal models (1, 4). It was also recently shown in osteoclast cultures that reduced extracellular pH resulted in nuclear translocation of NFATc1, a transcription factor with a central role in mediating downstream effects of RANKL in osteoclasts (13). What has not been established is whether there could be a role for extracellular pH sensing in the differentiation of osteoclasts.

The role of bone in regulating pH homeostasis is an important area of physiological and clinical interest (reviewed in Ref. 15). In experimental acidosis, renal H+ secretion does not increase to a level commensurate with increased acid production. Instead, balance is achieved by release of Ca2+ from bone and its secretion in the urine. Similarly, increased HCO3− that
results from reduced acid production is not secreted but is balanced by K+ and Ca2+. Recent work by Bushinsky and co-workers (16, 17) showed that metabolic acidosis suppresses bone formation by osteoblasts and, via a cyclo-oxygenase-dependent mechanism, increases RANKL synthesis, thereby increasing osteoclast differentiation and bone resorption. Work by Ludwig et al. (7) showing that protons bind OGR1 via extracellular histidines and that OGR1 was localized in osteoblasts and osteocytes led them to speculate that the effects of pH on the bone-forming side were mediated by OGR1.

Our evidence suggests that OGR1 plays a role at least as important on the bone-resorbing side. OGR1 is strongly expressed as an early event during osteoclastogenesis in vivo and in vitro. The levels of OGR1 mRNA peaked in vivo well before (i.e. 2 days) the peak in TRAP-positive osteoclasts, at a time when mononuclear osteoclast precursors express high levels of the chemoattractant CCL9 to facilitate migration and fusion (3). OGR1 protein was also highly expressed by RAW cells following exposure to RANKL in both mononuclear and multinucleated cells (Fig. 4). Thus, it appeared that OGR1 plays a role in osteoclast differentiation as well as having a possible role in osteoclast function. OGR1 is very likely to be strongly conserved functionally in mammals, because BLAST amino acid alignments show that rat and mouse share 96% identity and 98% similarity and mouse and human have 92% identity and 95% similarity.

Previous work has shown that other pH-sensing membrane proteins are expressed in osteoclasts, consistent with a role in osteoclast activity. Acid sensing ion channels 1, 2, and 3 were shown to be expressed in human peripheral blood mononuclear cells and to persist during their CSF-1- and RANKL-induced differentiation to osteoclasts (18). OGR1 was also shown to be expressed in RAW 264.7 cells at a single time point (4 or 5 days) after adding RANKL to the culture medium (13). Our experiments using anti-OGR1 antibody and siRNA to suppress OGR1 activity during the RANKL-induced differentiation process significantly reduced osteoclast differentiation (Figs. 5 and 6). This suggests that, in addition to bone resorption activity carried out by mature, terminally differentiated osteoclasts, extracellular pH sensing may also be important for osteoclast differentiation. OGR1 and its family members have been shown to bind and be regulated by bioactive lipids such as SPC, lyso-phosphatidylcholine, and psychosine (7, 8, 10). Whether these OGR1 ligands also play a role in osteoclast differentiation remains to be determined.

The differentiating osteoclast system may provide a useful tool in efforts to understand the mechanism of OGR1 action. Both proton- and lipid-induced signaling through OGR1 have been studied in a variety of cell systems in which OGR1 was transfected and overexpressed. The lone exception to date was a study done in primary aortic smooth muscle cells (19), in which OGR1 mediated cAMP and prostaglandin I2 accumulation in response to lowering pH. Transfected overexpression systems are valuable but also carry significant risk of artifactual G-protein associations due to abnormal stoichiometry of receptors to other cellular constituents (20). This may account for the disparate results reported. It is generally agreed that OGR1 stimulation leads to inositol triphosphate accumulation via Gq coupling as well as to increases in intracellular [Ca2+]i. Whether the findings of increased cAMP, indicative of Gs coupling, and the antagonism reported between lipids and protons (12) will be replicated in other systems is not yet determined. To unravel genuine biological effects and signaling via OGR1, BMC and RAW 264.7 cells will no doubt be useful tools, because OGR1 expression is inducible, occurs at physiological levels, and is coupled to cell-appropriate G-proteins.

It was recently reported that OGR1 played a role in pH sensing by osteoclasts and differentiated RAW cells (13). Those authors showed that OGR1 mRNA was up-regulated by RANKL in RAW cells and that the cells also became more sensitive to extracellular pH. Although the results are suggestive, much work remains to establish rigorously what the biological role for OGR1 is in mature osteoclasts. Additional studies may give further insight into the regulation of OGR1 as well as its activities. Inspection of the proximal 1 kb of the OGR1 promoter shows dozens of consensus binding sites for a variety of transcription factors, but only one weak NF-κB binding site. In the present study, we have shown that inhibition of OGR1 either by anti-OGR1 antibody or by siRNA has similar effects in blocking osteoclast differentiation in primary BMC and in
RAW cells. We also showed that OGR1 is highly expressed early during osteoclast differentiation in vivo, both of which indicate a role for OGR1 prior to their full differentiation into mature, bone-resorbing cells. This represents an important demonstration of a genuine physiological role for OGR1. Future studies should help to clarify the precise mechanism by which OGR1 acts during osteoclast differentiation and in the mature osteoclast.

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