Characterization of Regulatory Extracellular Vesicles from Osteoclasts

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Abstract

Extracellular vesicles (EVs), which include exosomes and ectosomes/microvesicles, have emerged as important intercellular regulators. EVs can interact with surface receptors of target cells and can transport luminal components, including messenger RNAs (mRNAs), microRNAs, and enzymes, to the cytosol of the target cell. Here, we show that hematopoietic cells grown in culture shed exosome-like EVs as they differentiate from preosteoclasts into osteoclasts. These EVs were between 25 and 120 nm (mean, 40 nm) in diameter determined by transmission electron microscopy. The exosome-associated markers CD63 and EpCAM were enriched in the isolated EVs while markers of Golgi and endoplasmic reticulum were not detected. Treatment of isolated hematopoietic cells with EVs did not affect their receptor activator of nuclear factor κ B–ligand (RANKL)–stimulated differentiation into osteoclasts. However, EVs from osteoclast precursors promoted 1,25-dihydroxyvitamin D₃–dependent osteoclast formation in whole mouse marrow cultures, and EVs from osteoclast-enriched cultures inhibited osteoclastogenesis in the same cultures. These data suggested that osteoclast-derived EVs are paracrine regulators of osteoclastogenesis. EVs from mature osteoclasts contained receptor activator of nuclear factor κ B (RANK). Immunogold labeling showed RANK was enriched in 1 in every 32 EVs isolated from osteoclast-enriched cultures. Depletion of RANK-rich EVs relieved the ability of osteoclast-derived EVs to inhibit osteoclast formation in 1,25-dihydroxyvitamin D₃–stimulated marrow cultures. In summary, we show for the first time that EVs released by osteoclasts are novel regulators of osteoclastogenesis. Our data suggest that RANK in EVs may be mechanistically linked to the inhibition of osteoclast formation. RANK present in EVs may function by competitively inhibiting the stimulation of RANK on osteoclast surfaces by RANKL similar to osteoprotegerin. RANK-rich EVs may also take advantage of the RANK/RANKL interaction to target RANK-rich EVs to RANKL-bearing cells for the delivery of other regulatory molecules.

Keywords: exosome, microvesicle, receptor activator of nuclear factor kappa B ligand, bone, osteoblast, osteoprotegerin

Introduction

Extracellular vesicles (EVs), which include exosomes and ectosomes/microvesicles, have gained attention as mediators of intercellular communication as well as ideal reservoirs for biomarker discovery (Bobrie and Théry 2013; Camussi et al. 2013; Lopez-Verrilli and Court 2013; Beninson and Fleshner 2014; Braccioli et al. 2014; Buzas et al. 2014; Kourembanas 2014; Loyer et al. 2014; Record et al. 2014). Receptors present on EVs can stimulate plasma membrane receptors of target cells (Kourembanas 2014). EVs are also internalized by target cells, and functional proteins, messenger RNAs (mRNAs), and microRNAs have been shown to be transferred into the cytosol of the target cell (Valadi et al. 2007). Because of their protein, lipid, and nucleic contents, which closely reflect the nature and physiological state of their cell of origin, EVs are also important sources of prognostic and diagnostic markers in body fluids (Kourembanas 2014).

The protein content of gingival crevicular fluid (GCF) from teeth undergoing external root resorption compared with control teeth was examined by our group (Rody et al. 2014). More than half of the proteins that were detected are found in the database ExoCarta, which catalogs proteins found in exosomes

(Keerthikumar et al. 2016). A number of the exosome-associated proteins changed significantly in quantity based on the root resorption state. This suggested that osteoclasts, osteoblasts, and other tooth or bone-associated cells may release exosomes or other EVs that might prove useful as biomarkers for root and bone resorption. These data also suggested that local regulation of bone remodeling might involve EVs.

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Bone remodeling and osteoclast formation are closely regulated by a signaling network involving receptor activator of nuclear factor κ B–ligand (RANKL), its receptor, receptor activator of nuclear factor κ B (RANK), and the competitive inhibitor of the RANKL–RANK interaction, osteoprotegerin (OPG) (Hofbauer et al. 2000; Boyce and Xing 2008). An ongoing dialogue occurs between osteoblasts, the bone-forming cells, and osteoclasts, the cells specialized for the resorption of bone (Boyle et al. 2003; Robling et al. 2006; Boyce and Xing 2008). In response to systemic signals, such as 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), and local signals such as mechanical stress, osteoblasts express RANKL on their surface, which binds RANK on the surface of monocytes. This binding triggers the differentiation of the monocyte into osteoclasts. Mature osteoclasts also express RANK and continue to require RANKL stimulation to resorb bone (Burgess et al. 1999). OPG is released by osteoblasts and binds RANKL, competitively inhibiting its binding to RANK (Bucay et al. 1998; Ominsky et al. 2008). Very recently, it was demonstrated that EVs containing RANKL are released from osteoblasts and stimulate osteoclast formation by engagement of RANK (Deng et al. 2015).

Our proteomic study of GCF suggested bone cells may release regulatory EVs, and the study by Deng and colleagues (2015) is consistent with this idea. Here, we show that EVs from osteoclasts and their precursors regulate osteoclast formation in $1,25(OH)_2D_3$ -stimulated mouse marrow and implicate EVs that are enriched in RANK in the inhibition of osteoclast formation.

Experimental Procedures

Reagents and Antibodies

$1,25(OH)_2D_3$ and minimum essential media, α modification (α -MEM) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Recombinant receptor activator of nuclear factor κ B–ligand (sRANKL) was produced in *Escherichia coli* as described previously (Hurst et al. 2004). Macrophage colony-stimulating factor 1 (CSF-1) was obtained from Peprotech (Rocky Hill, NJ, USA). Anti-RANK antibodies were obtained from EMD Millipore (Darmstadt, Germany; 04-1507), Novus Biologicals (Littleton, CO, USA; NB100-56508), and Biorbyt (Berkeley, CA, USA; Orb6560). Anti-EpCAM (D269-3) was obtained from MBL International (Woburn, MA, USA). Anti-Calnexin was from Novus (NB100-1965). Anti-GP96 (36-2600) was obtained from Life Technologies (Carlsbad, CA, USA). Secondary antibodies were obtained from Sigma-Aldrich. ExoQuick TM was obtained from System Biosciences (Mountain View, CA, USA). The Dynabeads antibody coupling kit was obtained from Life Technologies. DynaMag Spin was obtained from Invitrogen (Carlsbad, CA, USA).

Osteoclast Differentiation

Primary osteoclasts were grown from precursors obtained from Swiss-Webster mouse femora and tibiae (Toro et al. 2012). The University of Florida Institutional Animal Care and Usage

Committee approved all mouse protocols. Mice were sacrificed, bones were dissected, and marrow was expelled from bones using a syringe with α -MEM complete media (Sigma-Aldrich) plus 10% exosome-free fetal bovine serum (System Biosciences), 1% L-glutamine (Thermo Fisher Scientific, Waltham, MA, USA), and 1% penicillin/streptomycin/amphotericin B (Thermo Fisher). Cells were seeded in T75 flasks at a concentration of 1.5×10^6 cells/mL supplemented with 5 ng/mL recombinant murine Macrophage-Colony Stimulating Factor [CSF-1] (Peprotech, Rocky Hill, NJ, USA) and allowed to grow for 24 h at 37°C and 5% CO_2 . Nonadherent cells were removed, and 5.9×10^5 cells/mL adherent cells were seeded in 24-well plates or at 2.1×10^6 on 6-well plates. All cultures were supplemented with 10 ng/mL CSF-1 and 5 ng/mL soluble recombinant RANKL (sRANKL) (Hurst et al. 2004) to generate osteoclasts. To generate osteoclast precursors, sRANKL was omitted and cells were cultured for 3 d. To generate osteoclast-enriched cultures, cells were cultured for 5 or 6 d with α -MEM with 10% exosome free fetal bovine serum (System Biosciences) refreshed every 3 d.

$1,25(OH)_2D_3$ -stimulated mouse marrow was prepared as described previously (Holliday et al. 1995). Cervical dislocation was performed to sacrifice Swiss-Webster mice (8–20 g). Femora and tibia were dissected from the mice, and marrow was removed by cutting both bone ends, inserting a syringe with a 25-gauge needle, and flushing the marrow using α -MEM plus 10% fetal bovine serum (α -MEM D10). Marrow was washed and plated at a density of 4×10^4 cells/cm² on 24-well plates for 5 d in α -MEM D10 plus 10^{-8} M $1,25(OH)_2D_3$ plus exosomes as noted. Cultures were fed on day 3 by replacing half the media per plate and adding fresh $1,25(OH)_2D_3$. After 5 d in culture, osteoclasts appeared. These were detected as giant cells that stained positive for tartrate-resistant acid phosphatase activity (TRAP; a marker for mouse osteoclasts).

Isolation of EVs. All steps in EV isolations were done under sterile conditions. ExoQuick TC material from System Biosciences was used to isolate EVs from cultures of primary cells following the manufacturer's instructions. The final pellet, containing EVs and ExoQuick, was diluted 5-fold with phosphate-buffered saline (PBS) to induce the ExoQuick material to return to the soluble state. The samples were then spun at $200,000 \times g$ for 2 h in an Airfuge (Beckman Coulter, Brea, CA, USA) and the pellets were collected. Numbers of EVs were determined using the EXOCET kit (System Biosciences), which detects acetylcholinesterase activity, a general marker for exosomes. Assays were performed following the manufacturer's instructions.

Transmission electron microscopy. EVs were visualized as described previously (Théry et al. 2006). EV pellets were resuspended in 2% paraformaldehyde. The resuspended pellets were placed on Parafilm, and Formvar-carbon-coated electron microscope (EM) grids were floated atop the EVs for 20 min. Grids were then washed in PBS, transferred to 1% glutaraldehyde for 5 min, and then washed in deionized water 8 times. The grids were then contrasted and embedded by placing them on a drop of uranyl-oxalate solution and transferred to methyl

cellulose-uranyl acetate on ice for 10 min. The grids were blotted dry with filter paper and observed under an electron microscope. Colloidal gold tagging was also performed as described previously (Théry et al. 2006). EV pellets were resuspended in 2% paraformaldehyde. The resuspended pellets were placed on Parafilm and the Formvar-carbon-coated EM grids were floated atop for 20 min. Grids were washed in PBS, blocked with 0.05 M glycine in PBS and 1% BSA in PBS, and then washed in PBS again. The grids were then incubated with anti-RANK antibodies and gold-conjugated secondary antibodies. The grids were then washed with PBS, transferred to 1% glutaraldehyde for 5 min, and then washed in deionized water 8 times. The grids were contrasted with methyl cellulose-uranyl acetate on ice for 10 min. Last, they were blotted dry with filter paper and observed under the electron microscope. Electron microscopy was performed at the University of Florida, College of Medicine, Electron Microscopy Core Facility using a Hitachi (Tokyo, Japan) 7600 transmission electron microscope operated at 80 kV.

TRAP activity assay. TRAP activity was detected using the Leukocyte Acid Phosphatase kit (Sigma-Aldrich; cat. no. 387A-KT) following the instructions from the manufacturer. Osteoclasts were detected as staining positive for TRAP activity. TRAP⁺ cells were counted and classified as mononuclear, multinuclear (2–10 nuclei), or giant cells (≥ 10 nuclei) according to the number of nuclei present as described previously (Holliday et al. 1995; Ostrov et al. 2009).

Immunoblotting. EVs were loaded onto 4% to 20% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels, blotted to nitrocellulose. Immunoblots were performed by standard procedures using the Pierce DAB substrate kit (Life Technologies).

Immunoaffinity purification. Anti-RANK antibody (EMB/Milipore) or an anti-rat GST (Sigma) was dialyzed into PBS and then coupled to Dynabeads M-270 Epoxy Beads (Thermo Fisher Scientific) using the reagents provided in the Dynabeads Antibody Coupling Kit. The procedure was performed exactly as described in the manual provided with the kit. Immunoaffinity isolations were performed using a magnetic bead system. EVs (1×10^{10} in 500 μ L) in 20 mM Tris (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol, and protease inhibitors (F-buffer) were incubated for 2 h with 50- μ L beads; the supernatants were collected; and the beads washed thoroughly with F-buffer. Beads Bound material was eluted from beads with 100 mM glycine sulfate (pH 2.3). Densitometry of supernatants comparing RANK depletion with mock depletions was performed by separating 1×10^7 by SDS-PAGE, blotting with anti-RANK antibody (Biorbyt), and use of ImageJ (National Institutes of Health, Bethesda, MD, USA) to analyze the relative intensity of RANK bands.

Statistics. Counters were precalibrated for their ability to identify TRAP⁺ multinuclear cells, then blinded to treatment groups. Results are expressed as mean $\pm$ standard error. Samples were compared by 1-way analysis of variance (ANOVA)

Figure 1. Isolation and characterization of extracellular vesicles (EVs) from osteoclasts and their precursors. EVs were isolated from (A) osteoclast-enriched cultures and (B) osteoclast precursors and prepared for transmission electron microscopy. Arrows indicate examples of the most prominent vesicles observed in each preparation. Scale bar equals 100 nm. (C) The diameter of EVs was measured at their widest point. Total EVs measured were 444 from osteoclasts and 493 from preosteoclasts. (D) EVs from precursors and osteoclasts contained the exosomal markers EpCAM and CD63 and lacked gp96 and calnexin, endoplasmic reticulum proteins that are often found contaminating EV preparations.

and Student's *t* test using the program GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). *P* values < 0.05 were considered significant.

Results

Osteoclasts Release EVs during Osteoclastogenesis and during Bone Resorption

EVs were isolated from primary mouse marrow osteoclast-enriched cultures and precursors. Examination of the negative stained EVs showed that they were similar in size and morphology (Fig. 1A–C). EVs from osteoclasts and their precursors carried the exosome markers EpCAM (Runz et al. 2007) and CD63 (Pols and Klumperman 2009). EVs lacked gp96 and calnexin, endoplasmic reticulum markers that are common contaminants of EV preparations (Lasser et al. 2011) (Fig. 1D).

EVs from Osteoclasts and Precursors Regulate $1,25(\text{OH})_2\text{D}_3$ -Dependent Osteoclastogenesis in Mouse Marrow Cultures

To test whether osteoclast-derived EVs have the ability to regulate osteoclast formation directly, we added EVs (5×10^7)

Figure 2. Extracellular vesicles (EVs) from osteoclasts and preosteoclasts regulate osteoclastogenesis by a paracrine mechanism. **(A)** The hematopoietic population in mouse marrow was separated from mesenchymal/stromal cells, stimulated with colony-stimulating factor 1 (CSF-1) and receptor activator of nuclear factor κ B-ligand (RANKL) for 6 d in the presence of either 1×10^7 /mL (low) or 1×10^8 /mL (high) concentrations of EVs isolated from precursors or osteoclast-enriched cultures. EVs were added on day 1 and again on day 4 when the cells were fed. On day 6, the cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP) activity, and TRAP+ cells were counted as mononuclear, multinuclear (2–10 nuclei), or giant cells (≥ 10 nuclei). Four separate experiments were performed, $n = 4$ for each treatment group. Control averages were as follows: 557 mononuclear, 236 multinuclear, and 88 giant cells. No significant differences were detected. **(B–E)** Mouse marrow was cultured in the presence of $1,25(\text{OH})_2\text{D}_3$ and 5×10^7 EVs/mL from preosteoclasts or osteoclasts. Fresh EVs were added on the fourth day of the 6 d as the cells were fed. On day 6, the cells were fixed and stained for TRAP activity. **(B)** Control culture treated with vehicle, **(C)** culture treated with EVs from preosteoclasts, and **(D)** culture treated with EVs from osteoclasts. **(E)** Quantitative analysis of TRAP+ mononuclear, multinuclear (2–10 nuclei), and giant cells (≥ 10 nuclei). Combined results from 4 separate experiments are presented. $n = 18$ for controls, 12 for cells stimulated by EVs from osteoclast-enriched cultures and 11 for cells stimulated with EVs from precursors. Control averages were as follows: 964 mononuclear, 279 multinuclear, and 27 giant cells. Asterisks indicate different from control value, $P < 0.05$ by Student's t test.

isolated from preosteoclasts or osteoclasts to isolated mouse marrow hematopoietic precursors that were stimulated to differentiate with recombinant RANKL and CSF-1. No significant change in differentiation to multinuclear or giant osteoclasts was detected (Fig. 2A).

Next, we tested for regulation of osteoclast formation in $1,25(\text{OH})_2\text{D}_3$ -stimulated mouse marrow. $1,25(\text{OH})_2\text{D}_3$ stimulates expression of RANKL in mesenchymal stem cells, RANK in hematopoietic stem cells, and synchronous differentiation of osteoclasts and osteoblasts during a period of 6 d that is regulated by dialogue between cells of the osteoblasts and osteoclast lineages. EVs (5.0×10^7 EVs per mL) isolated from osteoclast precursors or osteoclasts were added to $1,25(\text{OH})_2\text{D}_3$ -stimulated mouse marrow on days 1 and 4 of the 6-d culture period. On day 6, the cells were fixed and stained for TRAP activity to detect osteoclasts. EVs from osteoclast precursors significantly stimulated the number of osteoclasts formed compared with controls (no EVs). In contrast, EVs from osteoclasts significantly reduced the number of osteoclasts formed compared with control values (Fig. 2B–E). Similar results were obtained using EVs isolated from RAW 264.7 cells to treat $1,25(\text{OH})_2\text{D}_3$ -stimulated mouse marrow (Appendix Fig. 1).

A Subset of EVs Released from Osteoclast-Enriched Cultures Contains RANK

Based on the recent study indicating RANKL-rich EVs from osteoblasts stimulate osteoclastogenesis, we examined osteoclast-derived EVs for the presence of RANK. Western blots were performed on EVs from osteoclasts or their precursors. RANK was detected at low levels in the precursors but at much higher levels in osteoclasts (Fig. 3A). To test whether RANK was on the surface of EVs, osteoclast-derived EVs were examined by immunoelectron microscopy after staining with anti-RANK antibodies. No primary antibody or an anti-RANK antibody (Biorbyt) that recognizes a cytosolic domain was used as a control. An anti-RANK antibody (EMB/Millipore) that binds an extracellular domain of RANK was used to label EVs. A secondary antibody carrying 10-nm gold particles was used to demonstrate the anti-RANK staining. A subset of EVs was heavily labeled with the EMB/Millipore anti-RANK antibody (Fig. 3B–G). While only 1 of every 32 EVs observed in random images from these grids was labeled, the labeled EVs typically had multiple gold particles associated (Fig. 3B).

RANK-Containing EVs Inhibit Osteoclastogenesis in $1,25(\text{OH})_2\text{D}_3$ -Stimulated Mouse Marrow

We next examined whether RANK-containing EVs are potential endogenous regulators of $1,25(\text{OH})_2\text{D}_3$ -stimulated mouse marrow. EVs were isolated from $1,25(\text{OH})_2\text{D}_3$ -stimulated mouse marrow on day 3 and day 6 of culture, blotted, and probed for RANK. After 3 d of differentiation, EVs contained a large amount of RANK. Surprisingly, on day 6, RANK was much less abundant in the EV isolate (Fig. 4A).

To test whether RANK-rich EVs contributed to the ability of osteoclast-derived EVs to inhibit osteoclastogenesis, we isolated

EVs from primary osteoclast-enriched cultures and then performed depletion experiments using immunomagnetic isolation with beads covalently coupled to anti-RANK antibody (EMB/Millipore) (Fig. 4B). EV preparations depleted of RANK were compared with extracts depleted with anti-GST, an irrelevant antibody; the amount of RANK was reduced 59% in the anti-RANK-depleted samples compared with the control by densitometry of blots (Fig. 4C).

EVs that were either RANK depleted or mock depleted were added to mouse marrow at a concentration of 5×10^7 EVs per well at day 1 and day 4 of a 6-d culture period. The removal of RANK-containing EVs significantly relieved the inhibition of osteoclastogenesis detected with mock-depleted EVs (Fig. 4D).

Discussion

For the first time, we have shown that osteoclast-enriched cultures release regulatory EVs. Osteoclast precursors released EVs that enhanced $1,25(\text{OH})_2\text{D}_3$ -dependent osteoclast formation in mouse marrow cultures. EVs from mature osteoclasts inhibited $1,25(\text{OH})_2\text{D}_3$ -stimulated osteoclastogenesis in the marrow cultures (Appendix Fig. 2). A subset of the EVs from mature osteoclast-enriched cultures

was rich in RANK, which led us to test whether it was the active agent in the EV fraction from osteoclasts. Depletion of RANK from isolated osteoclast-derived EVs significantly reduced their ability to inhibit osteoclast formation. RANK-containing EVs may inhibit osteoclastogenesis competitively by binding to RANKL (in the manner of OPG) and thus prevent stimulation of the RANK-signaling pathway in osteoclasts (Fig. 5). While studies suggest monomeric osteoprotegerin binds RANKL much tighter than monomeric RANK fragments, RANK in EVs presumably interact with RANKL as a trimer and form the high-affinity heterohexameric RANKL-RANK complex (Liu et al. 2010).

It is also possible that RANK-rich EVs may transfer their luminal cargo to the cytosol of osteoblasts and exert regulatory effects in that manner. The relatively low percentage of osteoclast-derived EVs released may favor use of RANK to target RANKL rather than simple competition. If so, the EVs could deliver proteins, mRNAs, and microRNAs that could serve to “instruct” osteoblasts, osteocytes, or other RANKL-expressing cell types (Fig. 5). We are currently examining the protein and nucleic acid composition of RANK-rich EVs, as well as testing their regulation of pure osteoblast cultures.

Figure 3. A subset of osteoclast-derived extracellular vesicles (EVs) has receptor activator of nuclear factor κ B (RANK) on its surface. **(A)** 1×10^7 EVs isolated from preosteoclasts and osteoclasts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), stained using Coomassie Brilliant Blue for total protein, or immunoblotted to nitrocellulose and probed with an anti-RANK antibody (Biorbyt). **(B)** EVs from osteoclast-enriched cultures were prepared for transmission electron microscopy (TEM) and probed with an anti-RANK antibody directed against an extracellular domain (EMB/Millipore), an antibody directed against the cytosolic domain (Biorbyt), or no primary antibody. All were then probed with gold-tagged secondary antibodies. Labeled or unlabeled EVs were counted, and the number of gold particles on labeled EVs was counted and tabulated. EVs from osteoclasts were prepared for TEM probed with either **(C)** an anti-RANK antibody (Biorbyt) that recognizes a cytosolic antigenic determinant or **(D–G)** an anti-RANK antibody from EMB/Millipore that detects an extracellular determinant. As shown in **(C)**, only background labeling was detected using the anti-RANK antibody directed against a cytosolic determinant, and EVs (arrows) were not labeled. In **(D)**, only a subset of EVs was labeled with the anti-RANK antibody that binds an extracellular determinant, but those were heavily labeled. Other examples are shown at higher magnification in **(E–G)**. The scale bar is equal to 80 nm (**C, D**) and 40 nm (**E–G**).

The results presented in this study complement a recent study that demonstrated that RANKL-containing EVs released from osteoblasts stimulate osteoclast formation (Deng et al. 2015). Taken together, these data support the hypothesis that RANK and RANKL shed in EVs may contribute additional layers of complexity to the RANKL/RANK/OPG network of regulatory interactions.

EVs from osteoclast precursors stimulated osteoclast formation in the marrow cultures. Although we do not yet know the mechanism, this result is potentially consistent with previous studies in which monocyte-derived EVs stimulated the osteogenic differentiation of mesenchymal stem cells (Omar et al. 2011; Ekstrom et al. 2013).

We suggest that EVs derived from osteoclasts or their precursors may prove useful for the treatment of bone disease or injury. In principle, either could be expanded from a patient's blood, and the EVs could be harvested and used therapeutically. For example, EVs from precursors may be useful to enhance bone remodeling necessary to heal fractures. EVs from osteoclasts may prove useful for the treatment of bone loss associated with osteoporosis. Understanding the mechanisms by which osteoclast-derived EVs regulate osteoclast formation

Figure 4. Receptor activator of nuclear factor κ B (RANK)-rich extracellular vesicles (EVs) are found in conditioned media from 1,25(OH)₂D₃-stimulated mouse marrow, and depletion of RANK-EV using anti-RANK immunomagnetic beads reduces the ability of EVs to inhibit osteoclastogenesis in 1,25(OH)₂D₃-stimulated mouse marrow. **(A)** 1×10^7 EVs from 1,25(OH)₂D₃-stimulated mouse marrow conditioned media collected after 3 d or 6 d in culture were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose, and probed with an anti-RANK antibody (Novus). Blot using an anti-EpCAM antibody is shown as a loading control. **(B)** Whole primary osteoclasts (100 μ g total protein), starting osteoclast EVs (5×10^7), unbound EVs (5×10^7), and EVs bound to anti-RANK (EMD/Millipore) beads (1×10^7) were separated by SDS-PAGE, blotted, and probed with an anti-RANK antibody (Biorbyt). **(C)** (5×10^6) mock-depleted and RANK (EMD/Millipore)-depleted EVs were separated by SDS-PAGE, blotted, and probed with anti-RANK antibody (Biorbyt). Bar graph of densitometry of 3 independent experiments comparing RANK with mock depletions is shown. Asterisk indicates significantly different from control by Student's *t* test, $P < 0.05$. **(D)** 1,25(OH)₂D₃-stimulated mouse marrow cultures were untreated (control) or treated with 1×10^7 EVs that were mock depleted or RANK depleted on days 1 and 4 of a 6-d culture. Cells were fixed and stained for tartrate resistant acid phosphatase activity, and mononuclear, multinuclear (2–10 nuclei), and giant cells (≥ 10 nuclei) were counted. Data were analyzed by analysis of variance, followed by Student's *t* test. Asterisks indicate significantly different from control by Student's *t* test, $P < 0.05$. Hash tags indicate significantly different from mock depleted by Student's *t* test, $P < 0.05$.

may provide a new set of therapeutic targets. Finally, machinery involved in the regulation may provide new biomarkers for bone resorption. Our initial interest in EVs derived from

osteoclasts was sparked by a study that identified many exosome-associated proteins in the GCF of patients undergoing root resorption (Rody et al. 2014).

We have not detected RANK in GCF (Rody et al. 2014; W.J. Rody Jr. and L.S. Holliday, unpublished data). Although RANK-rich EVs recovered from conditioned media increased over time in pure osteoclast cultures, in 1,25(OH)₂D₃-stimulated mixed cultures, little RANK was detected in conditioned media from mature cultures that are rich in both osteoblasts and osteoclasts. RANK-rich EV release may have been reduced due to regulatory signals present in the co-culture, or RANK-rich EVs may have been quickly taken up by target cells. The mechanism that explains the lack of RANK-EVs in conditioned media of co-cultures may also explain our failure to detect RANK in GCF.

In summary, we show that osteoclasts produce EVs, which have the capacity to regulate 1,25(OH)₂D₃-stimulated osteoclast formation in mouse marrow, a model for the in vivo bone microenvironment (Takahashi et al. 1988). RANK-rich EVs are novel inhibitors of osteoclastogenesis. Ongoing studies will test whether RANK-rich EVs function by simple competitive inhibition of the RANKL-RANK interaction, preventing the stimulation of RANK signaling in osteoclasts. RANK-rich EVs may also use the high-affinity RANKL-RANK interaction to target RANKL-expressing cells for the delivery of regulatory molecules that may include proteins, mRNAs, microRNAs, and noncoding RNAs.

Author Contributions

N. Huynh, L. VonMoss, contributed to conception, design, data acquisition, analysis, and interpretation, drafted the manuscript; D. Smith, I. Rahman, M.F. Felemban, contributed to data acquisition and interpretation, critically revised the manuscript; J. Zuo, contributed to data acquisition and analysis, critically revised the manuscript; W.J. Rody Jr, K.P. McHugh, contributed to conception and design, critically revised the manuscript; L.S. Holliday, contributed to

conception, design, data acquisition, analysis, and interpretation, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

Figure 5. Models for potential mechanisms of inhibition of osteoclastogenesis by receptor activator of nuclear factor κ B (RANK)–rich extracellular vesicles (EVs). Receptor activator of nuclear factor κ B–ligand (RANKL) on the surface of osteoblasts and other cells binds RANK on the surface of osteoclasts to stimulate osteoclastogenesis and bone resorption (bottom of osteoclast). RANK-rich EVs (red) are released from osteoclasts and may interact with RANKL on osteoblasts to competitively inhibit RANKL binding to RANK on osteoclasts (top of osteoclast). The RANK-RANKL binding interaction may target EVs to RANKL-containing cells (right of osteoclast). The bound EVs may subsequently fuse with the targeted cell delivering membrane elements and luminal components. This could occur either at the plasma membrane (as depicted) or after EVs are internalized into vesicular compartments.

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