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Osteoclast fusion is initiated by a small subset of RANKL-stimulated monocyte progenitors, which can fuse to RANKL-unstimulated progenitors

Noam Levaot [a,*], Aner Ottolenghi [a], Mati Mann [b], Gali Guterman-Ram [a], Zvi Kam [c], Benjamin Geiger [c,*]

[a] Department of Physiology and Cell Biology, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel
[b] Department of Biology and Biological Engineering, California Institute of Technology, Pasadena, CA, USA
[c] Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot, Israel

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ABSTRACT

Osteoclasts are multinucleated, bone-resorbing cells formed via fusion of monocyte progenitors, a process triggered by prolonged stimulation with RANKL, the osteoclast master regulator cytokine. Monocyte fusion into osteoclasts has been shown to play a key role in bone remodeling and homeostasis; therefore, aberrant fusion may be involved in a variety of bone diseases. Indeed, research in the last decade has led to the discovery of genes regulating osteoclast fusion; yet the basic cellular regulatory mechanism underlying the fusion process is poorly understood. Here, we applied a novel approach for tracking the fusion processes, using live-cell imaging of RANKL-stimulated and non-stimulated progenitor monocytes differentially expressing dsRED or GFP, respectively. We show that osteoclast fusion is initiated by a small (~2.4%) subset of precursors, termed "fusion founders", capable of fusing either with other founders or with non-stimulated progenitors (fusion followers), which alone, are unable to initiate fusion. Careful examination indicates that the fusion between a founder and a follower cell consists of two distinct phases: an initial pairing of the two cells, typically lasting 5–35 min, during which the cells nevertheless maintain their initial morphology; and the fusion event itself. Interestingly, during the initial pre-fusion phase, a transfer of the fluorescent reporter proteins from nucleus to nucleus was noticed, suggesting crosstalk between the founder and follower progenitors via the cytoplasm that might directly affect the fusion process, as well as overall transcriptional regulation in the developing heterokaryon.

Introduction

Physiological skeletal homeostasis is a well-coordinated process, regulated by the reciprocal actions of bone-forming osteoblasts and bone-resorbing osteoclasts [1,2]. The fine balance between these two activities is of critical physiological importance, since decreased bone resorption by osteoclasts leads to the formation of sclerotic bone, as seen in osteopetrosis, whereas excessive resorption drives the pathogenesis of osteoporosis, osteoarthritis, periodontal diseases, tumor metastasis into bone, and multiple congenital syndromes [3]. Thus, an understanding of the mechanisms that control the activity of osteoclasts is crucial to the diagnosis and treatment of many clinical conditions.

Osteoclasts are large, multinucleated cells derived from the monocyte/macrophage lineage, and their differentiation requires factors produced by marrow stromal cells, osteoblasts, osteocytes, or lymphocytes [4–7]. Two pivotal factors, necessary and sufficient to promote osteoclastogenesis, are the macrophage colony-stimulating factor (CSF-1), and the receptor activator of nuclear factor kappa B ligand (RANKL) [8,9]. CSF-1 supports osteoclast proliferation and survival, whereas RANKL induces activation of the osteoclast differentiation program, including activation of genes necessary for bone resorption, and genes required for fusion of monocyte progenitor cells. Cell fusion is an essential step in osteoclast development, shared only by a few other cell types such as skeletal myoblasts, trophoblasts in the placenta, and the egg and sperm [10–12]. Given its central physiological role, osteoclast fusion may serve as an attractive target for specific therapeutic intervention in bone diseases where the action of these cells is either excessive or deficient.

In recent years, several studies utilized mouse models and cell-culture approaches to address the molecular mechanisms underlying osteoclast fusion, identifying proteins such as DC-STAMP [13,14], CD47 [15,16], dynamin [17], OC-STAMP [18] and ATP6v0d2 [19] (see also review [20]), that are essential to this process. At the cellular level, however, the exact role of each of these molecules in the fusion process remains elusive.

In this study, we addressed the properties and heterogeneity of osteoclast monocyte progenitors participating in the generation of the polykaryon. For this purpose, we examined the kinetics and fusion

* Corresponding authors.
E-mail addresses: levaot@bgu.ac.il (N. Levaot), benny.geiger@weizmann.ac.il (B. Geiger).

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patterns of osteoclast progenitor cells, and developed a novel approach for tracking individual cells involved in the initiation of the fusion process, and its progression. Using RAW 264.7 cells tagged with different fluorescent markers, we demonstrated that only a small subset of "founder cells" within the population of RANKL-stimulated cells was capable of initiating the fusion process, following 48–72 h of stimulation. When RANKL-primed (i.e., cells incubated for 48 h with RANKL) RAW264.7 cells were mixed with unprimed RAW264.7 cells, we found that the unprimed RAW cells could fuse with a primed founder cell. Moreover, live-cell experiments indicated that as soon as a founder progenitor cell and a follower progenitor cell adhere to each other, they develop cytoplasmic communication, well before the fusion event becomes apparent. The significance of the heterogeneity of the progenitor cell population and the mode of cell–cell interaction involved in the fusion process are discussed.

Materials and methods

Generation of RAW264.7 clones expressing nuclear fluorescence proteins

To generate RAW264.7 clones, cassettes expressing nuclear fluorescence proteins DsRed and AceGFP containing nuclear localization signals from pDsRed2-NUC and pAcGFP-NUC, respectively [Clontech], were amplified by PCR, introducing NotI and ClaI restriction sites. The PCR product was cloned into the lentiviral vector pHAGE2-Full EF1a-DsRedExpress-IRES-ZsGreen-W (courtesy of Dr. Roi Gazit) by replacing the DsRED-IRES-Zs Green cassette, using the corresponding restriction enzymes. For the productions of virions, lentiviral vectors expressing the different nuclear fluorescent proteins were packaged in HEK293T cells, using the packaging plasmids pMD2.G and psPAX2 (Addgene). The virions were collected 48 h after transfection. RAW264.7 cells were infected for 48 h in the presence of 8 μg/ml polybrene (Sigma). Clones expressing different fluorescent nuclear proteins were selected in puromycin (3 μg/ml) for 3 days.

Isolation of bone marrow monocytes

Cells were harvested from the bone marrow of C57BL6 mice, as previously described [21]. Bone marrow cells were treated with ACK red cell lysis buffer (0.15 M NH_4Cl, 10 mM $KHC0_3$, 0.1 mM Na_2EDTA in distilled H_2O). Cells were plated on bacterial culture dishes in α-MEM and 10% FBS for three days in the presence of recombinant CSF-1 (20 ng/ml) (R & D Systems), and then stained and used in co-culture experiments.

Osteoclast fusion distribution analysis

To calculate the distribution of fusion events in which either mononucleated or multinucleated cells were involved, RAW264.7 cells expressing actin-GFP or BMMs were plated in wells of μclear 384-well plates at a density of 1000 cells per well, and cultured in α-MEM containing 10% FBS, 20 ng/ml murine recombinant CSF-1, and 20 ng/ml or indicated concentrations of RANKL. After 48 h in culture, medium was replaced, and cells were cultured for an additional 72 h, during which they were monitored by time-lapse microscopy. To compare fusion of mononucleated and multinucleated cells, fusion events were counted in a total of 60 movies from three independent experiments. A total of 1098 fusion events were counted in these experiments, and plotted against time. To compare mononucleated cell fusion in the presence of different concentrations of RANKL, fusion events were counted in ten movies for each RANKL concentration. The numbers of fusion events/field/cell were also quantified. To monitor founder cell distribution, RAW264.7 clones expressing nuclear AceGFP were plated in 250 μm-wide wells of CytoCapture dishes (Mobitec), by plating a total of 100,000 cells per dish. Cell density in each well ranged between 12 and 120 cells per well. After 72 h of culture in the presence of CSF-1 and RANKL, cells were imaged, and the total number of cells and multinucleated cells with three or more nuclei within each well was counted.

Founder-follower co-cultures

For generation of "founder"-enriched cultures, RAW264.7 clones expressing dsRED were plated in wells of μclear 384-well plates, at a density of 1000 cells per well. The cells were cultured in α-MEM containing 10% FBS, and 20 ng/ml murine recombinant CSF-1 with 20 ng/ml RANKL (Founders), or without RANKL (Control). RAW264.7 clones expressing nuclear AceGFP were cultured in α-MEM containing 10% FBS and 20 ng/ml murine recombinant CSF-1 without RANKL; these served as followers. After 50 h of incubation, follower cells were lifted off the plates, and 1000 cells were transferred to wells containing either DsRED founder or control cells. The co-cultured cells were cultured in the presence of CSF-1 and RANKL for a duration of 12 h, during which they were monitored by time-lapse microscopy.

For BMM co-culture experiments, BMMs were separated into two groups. One group was stained with the cytoplasmic dye PKH67, and the second group with Cell Vue Claret dye, according to manufacturer's protocols (Sigma). Cell Vue Claret-stained BMMs were plated in wells of μclear 384 well plates at a density of 1000 cells per well, and cultured in the presence of 20 ng/ml CSF-1, with 20 ng/ml RANKL (Founders) or without it (Control). BMMs stained with PKH67 dye were cultured only in the presence of CSF-1, and served as the follower group. Founder and Control BMMs were cultured for 72 h, after which 1000 follower cells were added to each well. The co-cultured cells were cultured in the presence of CSF-1 and RANKL for 12 h, during which they were monitored by time-lapse microscopy.

Microscopy

Movies and fixed cell data were acquired with a live imaging system, consisting of an inverted IX81 microscope equipped with 20×/0.75 NA objectives (Olympus) and with a temperature-controlled box using CellSens software (Olympus). Nuclear fluorescence transfer was calculated in the following way: nuclei segmentation and the measurement of total nuclei intensity were performed by means of ImageJ software. The initial green fluorescence in the red nuclei was set as "background," and subtracted from the green fluorescence readings at each time point. To normalize the results to the initial fluorescence level of the green nuclei, the green fluorescence readings in the red nuclei at each time point were divided by the initial green fluorescence of the green nuclei, minus the background green fluorescence.

Immunofluorescence

Three clones of RAW264.7 cells were seeded on coverslips and induced to differentiate using the conditions outlined above. Cells were fixed at 48 h of differentiation in 4% paraformaldehyde, permeabilized in PBS containing 0.1% Triton X-100 and blocked with PBS containing 2% FBS for 1 h. NFATc1 expression was detected using overnight incubation in NFATc1 mAb (1:100, Santa Cruz Biotechnology) and four hours' incubation with Cy2-conjugated antimouse secondary antibody (AffiniPure, Jackson). Nuclei were counterstained with 4,6-diamidino-2-phenylindole (Sigma-Aldrich). Nuclei and cytoplasmic fluorescence intensities were quantified using ImageJ, and normalized to background levels.

Statistical analysis

The probability picking founders in a cell population is governed by Poisson statistics, but can be well approximated by normal (Gaussian) statistics for large numbers of cells per sample (50 ± 20 for our experiment). We counted the initial number of cells and listed the number of fused cells in 142 samples. We plotted the distribution of x, the