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CHAPTER 5

Migration dynamics of osteoclasts and their precursors during differentiation

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Abstract

Osteoclasts are specialized cells with the unique capacity to resorb bone. The formation of these cells is a multistep process in which migration is essential for both the precursors and the osteoclast itself. Yet, surprisingly little is known about the dynamics of cell migration throughout the process of osteoclast formation. In this study we investigated migratory behavior of osteoclast precursors at different time points. Human peripheral blood mononuclear cells (PBMCs) were cultured with the osteoclastogenic cytokines macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-KB-ligand (RANKL). The cultures were analyzed by live cell microscopy during 96 hours, both after one week and after two weeks of culture. At an early stage the cells migrated further away from their initial position compared to the later stage. At the two weeks time point the cells were motile, but did not travel long distances. We next monitored isolated osteoclasts and showed for the first time that osteoclasts can undergo fission and generate functional multinucleated compartments as well as compartments that contained apoptotic nuclei. Together, these findings suggest important differences in the dynamics of cell migration during osteoclastogenesis: First, the osteoclast precursor explores the environment in search for fusion partners whereas in a later stage the osteoclast precursor moves in a much more localized area, possibly preparing for an interaction with neighboring cells. The interesting observation of dividing osteoclasts could suggest that the osteoclast divides into several multinucleated cells in order to simultaneously control bone resorption at different sites and shed apoptotic nuclei.

Introduction

Bone metabolism is based on bone formation and bone resorption, two major cellular processes that are regulated by specialized cells. Osteoblasts are the cells responsible for matrix deposition and the final formation of bone whereas osteoclasts are involved in bone degradation \(^\text{1-4}\). These latter cells are multinucleated, tartrate resistant acid phosphatase (TRACP)-positive and they are formed by fusion of osteoclast precursors \(^5\). The formation of such bone resorbing osteoclasts is a multistep process which includes recruitment of the precursors, their adhesion to bone lining cells/osteoblast-
like cells and the subsequent migration to the bone surface where they ultimately fuse to form an osteoclast.

Cell-cell interaction between osteoblast-like cells and osteoclast precursors is crucial in these processes and it has been shown that this interaction significantly induces gene expression and highly promotes the formation of osteoclasts. The resultant of the cell-cell interaction, being osteoclast formation, can be mimicked in vitro by the addition of the cytokines macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear kappa b ligand (RANKL) to the osteoclast precursors.

Not only during the formation of the osteoclasts but also at a later stage are the motile properties very important. Active bone resorbing osteoclasts produce a trail of resorption pits, indicating that also at this stage migration is essential.

Along with migration the cytoskeleton is rearranged. This involves polymerization of actin, the primary component of the part of the cytoskeleton involved in migratory movements, and the formation of membrane extensions called lamellipodia. After a contractile force has been generated inside the cell, adhesion is released locally at the trailing edge and the cell moves forward. Furthermore, it has been described that stimulators of osteoclastogenesis such as M-CSF can induce migration of both the osteoclast precursor and the osteoclast; a process mediated by Rac signaling.

Despite this insight in migration of osteoclast precursors and osteoclasts, surprisingly little is known about the dynamics of this process during osteoclastogenesis. In the present study we followed osteoclast precursors during different time intervals using live imaging and we analyzed their migratory behavior. Concomitantly, we examined the changes in morphology of the cells between different time points. Finally, we studied native mature osteoclasts and their migratory activity in a co-culture with osteoblast-like cells.

**Materials and methods**

**Cell cultures**

Peripheral blood mononuclear cells (PBMCs) were isolated according to earlier described protocols. Cells were cultured in 2-well chamber slides (Nunc, Roskilde, Denmark) in the absence or presence of 25 ng/ml
recombinant human M-CSF (R&D Systems, Minneapolis, MN) and 40 ng/ml recombinant human RANKL (PeProtech, Rocky Hill, NJ) in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco, Paisley, UK) containing 10% fetal calf serum (FCS; Hyclone, Logan, UT) and 1% antibiotics (100 U/ml penicillin, 100 g/ml streptomycin, and 250 ng/ml amphotericin B [Antibiotic antimycotic solution, Sigma St. Louis, MO]).

**Time lapse microscopy and image processing**

Cells were imaged using a Leica IR-BE (Leica Microsystems GmbH, Germany) inverted wide field microscope at 37ºC in an atmosphere containing 5% CO₂. Phase contrast images were acquired at 10 min time intervals using a 40x objective. Images were processed and analyzed using custom-made software and Image Pro Plus (Mediacybernetics, Carlsbad, CA, USA).

**Actin and CD44 staining**

Osteoclast plasma membranes were visualized with a CD44 staining as described before. Mouse bone marrow cells were seeded on cortical bone and allowed to mature for 8 days in the presence of M-CSF and RANKL, as described earlier. Slices were washed in PBS, fixed in 4% PBS buffered formaldehyde for 5 min, and subsequently washed in PBS. Nonspecific binding to cells was blocked for 30 min with 10% normal goat serum (Vector Laboratories, Burlingame, CA) followed by an overnight incubation at 4°C with rat anti-mouse anti-CD44 antibody IM7.8.1 (Cedarlane Laboratories Ltd., Burlington, ON L7L 5R2, Canada). Subsequently, slices were washed three times with PBS and bound antibody was visualized with goat-anti-rat-Alexa 647 conjugated antibody (Invitrogen/Molecular Probes, Carlsbad, CA) which was applied for 60 min. Following three PBS washes, F-actin was stained according to a method described previously using Alexa 488-phalloidin (Invitrogen/Molecular Probes). Finally, nuclei were stained with propidium iodide (Sigma-Aldrich, St. Louis, MO). Image stacks were generated using confocal laser scanning microscopy (Leica) using an argon laser (Alexa 488 and propidium iodide) and a helium laser (Alexa 647).

**Native osteoclasts**

Native osteoclasts were isolated from New Zealand White rabbits of 5 days old. Long bones were dissected and collected in 10 ml alpha Minimal
Essential Medium (α-MEM, Gibco, Paisley, UK) with 1% antibiotics but without fetal calf serum (FCS). The bones were cut for 10-15 min into very small fragments. This homogenate was transferred to a 50 ml tube. 35 ml of α-MEM without FCS and with 1% PSF was added and the pieces were gently shaken for 30 seconds to free the osteoclasts from the bone. After 90 seconds of sedimentation the supernatant was collected. The last part of the procedure was repeated once more with 25 ml of α-MEM. Supernatants were collected and centrifuged for 2 min at RT at 700 rpm. The pellet, containing the osteoclasts, was washed once with 50 ml α-MEM containing 5% FCS (Hyclone, Logan, UT), subsequently centrifuged and collected in 10 ml of α-MEM containing 5% FCS and 1% PSF and finally seeded in 25cm² costar (Corning Inc., Corning NY) culture flasks. After 48 hours at 37°C, 5% CO₂ the osteoclasts were monitored for 80 hours by time lapse microscopy as mentioned above.

Statistical analysis
An unpaired t-test was performed to analyze significant differences between the different time points in figure 3 and 4. Means were considered to be significantly different when p < 0.05.

Results

The motility of osteoclast precursors during osteoclastogenesis
In order to analyze the motility of osteoclast precursors during osteoclastogenesis, we monitored mono-cultures of human PBMCs in the presence of M-CSF and RANKL at different time points during osteoclast formation. Based on their morphology, two subpopulations could be distinguished after one week of culture. The first group comprised about 42% of all cells; they were round, did not move from one site to another and increased their cytoplasmic area over time. Although not migrating, these cells were highly motile and interacted with neighboring cells by touching them (Fig. 1).
Osteoclast precursors after one week of culture are highly motile and actively migrate. Human PBMCs were cultured on chamber slides in the presence of M-CSF and RANKL for ten days and followed from day 7 until day 10. Subpopulations of round (e.g. cells 1 and 3) and elongated cells (e.g. 2 and 4) were observed. Migration dynamics were studied during this time interval.

Besides this group of cells, there was a second population (58 %) consisting of cells which were initially round but became elongated and formed a leading edge and lamellipodia. When elongated, the cells could reach a length of 100 µm, up to ten times their initial size. They actively migrated in different directions. Interestingly, there was hardly any direct interaction with other cells during this process.

We next analyzed the migratory dynamics of osteoclast precursors after two weeks of culture. At this stage osteoclast precursors are more differentiated towards the osteoclast lineage and some of them will eventually fuse to form multinucleated osteoclasts. In contrast to the situation after one week, most of the cells were flattened at this time point and a protruding leading edge was less pronounced. The cells hardly moved away from their original position but were still highly motile and seemed to communicate with/touch each other via their plasma membranes that attached and detached to each other. A minority of cells (18%) was
elongated and a few multinucleated osteoclast-like cells were already present at this time point (Fig. 2).

Figure 2. Osteoclast precursors after two weeks of culture are highly motile and mainly interact with neighboring cells. Human PBMCs were cultured on chamber slides in the presence of M-CSF and RANKL for 17 days and followed from day 14 until day 17. Subpopulation of round and elongated cells were observed. Migration dynamics were studied during this time interval.

Migration properties of osteoclast precursors
To get a better insight into the behavior of osteoclast precursors at the different stages during the culture period, we analyzed the cells during a period of 96 hours (both after one week and two weeks of culture) and assessed the mean distance a cell migrated away from its original position and the total distance it traveled throughout this 4 day time interval. An example of migration tracks is shown in Fig. 3A. The analysis of these parameters revealed that in an early stage (after one week) osteoclast precursors migrated further away from their initial point compared to a later stage (Fig. 3B). The mean distance from the origin was 93.9 µm ± 9.0 and 55.6 µm ± 4.7 (mean ± SEM), respectively.
Next we analyzed the total distance a cell migrated in the time interval studied (4 days), regardless the direction of movement and the distance from the origin. Fig. 3 shows that the total distance migrated was more at a later stage (850 µm ± 24.8) compared to that at an early stage (604 µm ± 37.0) (Fig. 3C).

**Figure 3.** Osteoclast precursors have different migration properties depending on their differentiation stage in osteoclast formation. Human PBMCs were cultured in chamber slides in the presence of M-CSF and RANKL for 10 or 17 days. A. Migration tracks of osteoclast precursors after 14 days of culture are depicted. Every ten minutes the position of the cell was determined. B. The distance from the original position per cell movement was analyzed in a time span of four days from day 7 until day 10 (75 cells analyzed) and day 14 until day 17 (67 cells analyzed). The mean distance is depicted. C. The total distance a cell migrated was analyzed in a time span of four days from day 7 until day 10 (66 cells analyzed) and day 14 until day 17 (78 cells analyzed). The mean total distance is depicted. c: p < 0.001. The movements of ± 70 cells were analyzed.

**Mature osteoclasts can undergo fission**

Isolated osteoclasts and co-isolated osteoblast-like cells were cultured and monitored for four days. Initially, the osteoblast-like cells were encircling the osteoclast, leaving a cell-free space between them and the osteoclast. The osteoclast then moved in this enclosed area and its plasma membrane
seemed to quickly attach and detach to the membranes of neighboring osteoblast-like cells. Osteoblast-like cells proliferated over time and entered the cell-free area. Eventually the osteoclast was covered with osteoblast-like cells and they migrated underneath this confluent layer of osteoblasts.

Interestingly, we observed that individual multinucleated osteoclasts could separate into two or more cells. The initial osteoclast was flattened, pancake-like and seemed firmly attached to the surface (Fig. 4A). Prior to its fission into two multinucleated cells, the shape of the osteoclast became capricious and two parts, only connected via a very thin cellular extension that formed a bridge-like structure, were formed (Fig. 4B). These parts were highly motile and migrated away from each other, hereby elongating the tubular connection between them (Fig. 4C-D). Finally, the connection collapsed which resulted in the generation of two separate multinucleated osteoclasts (Fig. 4E).

![Figure 4](image)

**Figure 4.** Mature osteoclasts can undergo fission and have the capacity to migrate under a confluent osteoblast layer. Rabbit osteoclasts were isolated together with co-isolated osteoblast-like cells. These cells were cultured overnight on culture dishes and cell movement was analyzed using live cell microscopy of time lapse cinematography. A. Mature osteoclasts are surrounded by osteoblast-like cells. B-D. Tubular structures (arrows) are formed by the osteoclast and osteoclast “islands” are separated. E. Osteoclasts are covered by osteoblast-like cells.

Osteoclasts that divided into more than two cells also used this mechanism. The separation of the new cells could be either simultaneously or sequentially, meaning that multinucleated osteoclasts could split directly in three or e.g. in two and then again in two.
Interestingly, this phenomenon was also seen with mouse osteoclasts on cortical bone slices. Osteoclasts matured from mouse bone marrow cells on cortical bone slices in the presence of M-CSF and RANKL and displayed groups of nuclei separated by very thin tubular structures (Fig. 5). Osteoclasts existed where groups of nuclei were all connected to one another. Furthermore, the presence of actin rings in some osteoclast compartments indicated that these osteoclast “islands” can display bone resorbing activity (Fig. 5). Next to this, in some osteoclast islands we observed nuclei which were reduced in size and seemed to be apoptotic.

Figure 5. Mature osteoclasts can get rid of apoptotic nuclei by fission. Mouse osteoclasts were formed from bone marrow cells and cultured on bone slices in the presence of M-CSF and RANKL and stained for actin (green) and CD44 (blue). Nuclei were stained with propidium iodide (red). An osteoclast with three multinucleated compartments connected with tubular structures (arrows) is depicted. An osteoclast compartment with apoptotic nuclei and without clear actin rings was observed (2). For color figure see p.163.

Discussion
Cell migration is essential during osteoclast formation. Osteoclast precursors attach to the bone lining cells, migrate to the bone surface and ultimately fuse into multinucleated osteoclasts. These bone resorbing cells subsequently migrate to the site where bone has to be resorbed. This study is the first to show differences in migration dynamics of osteoclast precursors at different stages of their differentiation. At an early stage the elongated cells actively migrate in different directions and seem to explore
the environment whereas at a later stage their migration radius is small and they primarily interact with neighboring cells.

The early stage corresponds to the differentiation stage wherein osteoclast precursors, after adhesion to osteoblast-like cells, need to migrate to the surface to search for possible fusion candidates. These potential fusion partners can then further differentiate in close vicinity to each other, this way facilitating cell-cell interactions, and eventually fusion. This could explain that at a later stage the osteoclast precursors (again round) are still highly motile but do not travel far from their site of attachment where they closely interact with each other. These interactions are characterized by a quick, short touching of a cell’s plasma membrane with the ones of neighboring cells and were established abundantly in a certain time interval. Together, this suggests that such a hyper movement is required for fusion to take place. The elongated cells which are observed in this later stage do not interact with neighboring cells, probably because they are not yet fully differentiated.

A molecule possibly responsible for the migratory behavior could be M-CSF. It has been shown that M-CSF stimulates migration of osteoclasts\(^{10,11,17,18}\). Moreover, M-CSF is considered to play a major role in the differentiation towards the osteoclast lineage\(^{19-24}\).

The migration of osteoclasts has been studied in relation to their bone resorbing activity and several mechanisms that are involved in these processes are described in the literature\(^{17,25-28}\). The tubular structures we observed between osteoclasts have strong similarities with the so called connecting and coordinating tubules described by Vesely et al. They have shown the presence of osteoclast satellites connected to osteoclasts via these tubular parts and have shown that these satellites, or compartments, are able to resorb bone\(^\text{29}\). However, the observation of osteoclasts that have the capacity to separate into more than one highly motile multinucleated (smaller) osteoclast has not been reported before. Could it be that osteoclasts divide in order to increase the number of resorbing cells when bone has to be resorbed at several sites simultaneously? It has been demonstrated that migrating osteoclasts do not resorb bone and vice versa\(^\text{17}\). It is possible that, when a fast turnover is required and only a few osteoclasts are present, these cells receive signals to undergo fission and thus quickly increase the osteoclast population. It could also well be that mature osteoclasts, after resorption, split off defective/apoptotic nuclei. Our
confocal images (Fig. 5) show that the nuclei in the smaller compartment (Fig 5. No. 2) are smaller and fragmented. In addition, no obvious actin rings were observed in this compartment. This strongly suggests a way for the original multinucleated cell to get rid of non-functional nuclei. It is, however, unclear at the present time what the mechanisms are behind this phenomenon. Therefore, further investigation on the fission of osteoclasts with its concomitant allocation of nuclei into various compartments is required and can reveal important pathways in post-fusion differentiation of osteoclasts.

Another interesting finding was the ability of osteoclasts to migrate under a confluent layer of osteoblasts. This was also demonstrated by Vesely et al. and called the ability of underlapping of other cells by osteoclasts 29. Is this an extra mechanism to facilitate osteoclast activity? It is plausible that this capacity to move underneath a cell layer is needed when bone has to be resorbed at places directly after bone lining cells had cleaned the bone surface of non-mineralized collagen 30. Osteoclasts at the bone surface could then migrate under the bone lining cell/osteoblast layer to resorb the bone. Andersen et al. recently demonstrated that osteoclasts on the bone surface of bone remodeling sites are covered by a canopy of flat, osteoblast-like cells. This canopy also seemed to be associated with the presence of capillaries, indicating vascularization in the recruitment of osteoclasts and bone forming osteoblasts 31.

Taken together, the data presented in this study provide new insights in the dynamics of cell migration during osteoclast formation and show for the first time that mature osteoclasts can undergo fission and separate themselves into functional smaller ones. We propose the following sequence of events during osteoclast formation: (1) the adhesion of osteoclast precursors to bone lining cells, (2) the retraction of the bone lining cells and migration of the osteoclast precursors towards the bone surface, (3) further differentiation of osteoclast precursors and their fusion, (4) the fusion of different multinucleated cells to form a giant cell, (5) migration of osteoclasts at the bone surface and underneath a layer of bone lining cells to resorb bone, (6) the optimization of osteoclast activity by fission of the osteoclast in more functional osteoclast compartments as well as the separation of compartments that contain non-functional nuclei.
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