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Published in:
Journal of Bone and Mineral Research

DOI:
10.1002/jbmr.3091

Publication date:
2017

Document version
Final published version

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Citation for published version (APA):

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Download date: 05. May. 2021
Coupling of Bone Resorption and Formation in Real Time: New Knowledge Gained From Human Haversian BMUs

Nicolai Ernlund Lassen,1 Thomas Levin Andersen,1,4 Gro Grunnet Pløen,1,8 Kent Søe,1 Ellen Margrethe Hauge,2 Søren Harving,3 Gete Ester Toft Eschen,4 and Jean-Marie Delaisse1

1Clinical Cell Biology, Institute of Regional Health Research, University of Southern Denmark, Vejle/Lillebaelt Hospital, Vejle, Denmark
2Department of Clinical Medicine, Aarhus University, Aarhus, Denmark
3Department of Orthopaedic Surgery, Aalborg University Hospital, Aalborg, Denmark
4Department of Plastic Surgery, Aarhus University Hospital, Aarhus, Denmark

ABSTRACT
It is well known that bone remodeling starts with a resorption event and ends with bone formation. However, what happens in between and how resorption and formation are coupled remains mostly unknown. Remodeling is achieved by so-called basic multicellular units (BMUs), which are local teams of osteoclasts, osteoblasts, and reversal cells recently proven identical with osteoprogenitors. Their organization within a BMU cannot be appropriately analyzed in common histology. The originality of the present study is to capture the events ranging from initiation of resorption to onset of formation as a functional continuum. It was based on the position of specific cell markers in longitudinal sections of Haversian BMUs generating new canals through human long bones. It showed that initial resorption at the tip of the canal is followed by a period where newly recruited reversal/osteoprogenitor cells and osteoclasts alternate, thus revealing the existence of a mixed “reversal-resorption” phase. Three-dimensional reconstructions obtained from serial sections indicated that initial resorption is mainly involved in elongating the canal and the additional resorption events in widening it. Canal diameter measurements show that the latter contribute the most to overall resorption. Of note, the density of osteoprogenitors continuously grew along the “reversal/resorption” surface, reaching at least 39 cells/mm on initiation of bone formation. This value was independent of the length of the reversal/resorption surface. These observations strongly suggest that bone formation is initiated only above a threshold cell density, that the length of the reversal/resorption period depends on how fast osteoprogenitor recruitment reaches this threshold, and thus that the slower the rate of osteoprogenitor recruitment, the more bone is degraded. They lead to a model where the newly recognized reversal/resorption phase plays a central role in the mechanism linking osteoprogenitor recruitment and the resorption-formation switch. © 2017 American Society for Bone and Mineral Research.

KEY WORDS: BONE HISTOMORPHOMETRY; BONE REMODELING; OSTEOCLAST; OSTEOBLAST; HUMAN

Introduction
Coupling of osteoclastic bone resorption to osteoblastic bone formation is a key to preserve bone architecture and strength. It is more and more regarded as a major step of the remodeling cycle and is receiving increasing attention. A number of regulators of coupling have been identified—both positive and negative ones—and interestingly from a mechanistic point of view, many of them appear to depend on the presence of osteoclasts.(1,2) At the bone tissue level, coupling was found to require recruitment of osteoprogenitors onto the eroded surface.(3–5) Immunostainings with specific markers(3,6) showed that these osteoprogenitors correspond with the cells that are defined as “reversal cells” in the standard bone nomenclature(7) and that were first mentioned by Baron.(8) Thus, reversal/osteoprogenitor cells deserve attention when seeking to understand the overall mechanism linking osteoclastic resorption and initiation of bone formation during bone remodeling.

However, the successive cellular events occurring on the eroded surfaces between bone resorption and initiation of bone formation are difficult to assess as a continuum and remain mostly uninvestigated. One reason is that sections used in
standard bone histomorphometry rarely happen to be parallel to the operational axis of the cell team operating within a local remodeling unit (also called basic multicellular unit [BMU]). Histological sections mostly randomly hit remodeling events occurring in distinct BMUs. Assessments of these events are routinely used to calculate the average overall values of resorption, reversal, and formation parameters in a given biopsy but are done independently of the BMU to which they belong. It has been found convenient to summarize these average values in a diagram representing a virtual BMU, where the remodeling events are shown as three single consecutive steps aligned according to their theoretical sequence: resorption, reversal, and formation. These diagrams may give the illusion that we know the spatiotemporal organization of the cell team operating within a BMU, but they represent only a model and not a picture captured from a real BMU. One should thus be aware that the dynamics of the cell populations meeting on the eroded surfaces remain to be investigated and that they appear important to investigate because they are expected to contribute to the important decision whether the bone resorbed at a given site should undergo further resorption or be left unreconstructed or be replaced by new bone formation.

The present investigation addresses the cell organization on the eroded surface within individual BMUs. It takes advantage of the fact that the orientation of intracortical BMUs is almost parallel to the long axis of the diaphysis of long bones. The remodeling space created by an intracortical BMU appears as a tunnel that the BMU opens and then closes down to a narrow canal (known as Haversian canal) by depositing concentric layers of bone matrix (known as an osteon). Longitudinal sections of diaphyses thus sometimes happen to pass almost exactly at the level of the operational axis of a BMU, and adjacent serial sections then allow the evaluation of the organization of this BMU in 3D. Importantly, BMU sections obtained in this way show the successive remodeling steps in their natural order, and these steps can thus be analyzed as a functional continuum. The present analysis considers only BMUs generating new Haversian canals (as assessed in serial sections) and where opening and closure of the resorption tunnel occur almost symmetrically around its central axis as typically shown in textbooks. This symmetrical arrangement offers a situation where the remodeling events are easier to interpret. We expect that the basic knowledge gained by these observations will later help to address more complex situations, including those leading, for example, to pathological porosity.

**Materials and Methods**

**Patients and biopsy specimens**

The histological analysis was conducted on decalcified paraffin-embedded bone specimens collected from the proximal femur of 9 patients during corrective surgery for coxa valga (6 females and 3 males, aged 10 to 20 years, mean age 13.9 years) and from the fibula of 10 patients during jaw-reconstructive surgery (3 females and 7 males, aged 41 to 73 years, mean age 55.4 years). Five- to 10-mm-thick transverse bone discs from the left and right femur or from the fibula of each patient were cut along the longitudinal axis into 6 to 8 smaller bone pieces. These bone pieces were fixed in 4% paraformaldehyde (PFA) for 2 days and decalcified in 15% EDTA with 0.4% PFA for 60 days. The decalcified cortical bone specimens were then dehydrated and paraffin-embedded, orienting them in such a way that the Haversian canals within the cortical bone were cut longitudinally. The study was approved by the Danish National Committee on Biomedical Research Ethics (Project-ID: S-2012-0193).

**Sectioning and selection of Haversian canals with remodeling activity**

Series of 20 to 60 adjacent 3.5-μm-thick longitudinal sections were cut from 120 of the 200 collected cortical bone specimens. Because the number of Haversian canals showing remodeling activity is quite low, section numbers 5, 15, 25, and so on were either Masson Trichrome stained or immunostained in order to identify the series of consecutive sections containing both a cutting cone generating a new canal and the beginning of the closing cone. The canals with a very asymmetrical distribution of remodeling activity were deselected because they are more difficult to analyze. The intervening unstained consecutive sections of the selected Haversian canals were immunostained and in situ hybridized for different markers. This allowed the characterization of the cells colonizing the eroded surfaces and the generation of 3D reconstructions showing the sequential steps involved in the remodeling process.

**Immunohistochemistry**

The sections were single or double immunostained as described previously. In short, the consecutive sections used for the 3D reconstructions were immunostained with rabbit anti-Cathepsin K (CatK; AP08851PU-N, Acris, Herford, Germany) antibodies. Some of the consecutive sections used to characterize the eroded surfaces were double immunostained with mouse IgG1 anti-CD56 antibodies (clone 56C04, Thermo Fisher Scientific, Waltham, MA, USA) combined with either mouse IgG2b anti-tartrate-resistant acid phosphatase (TRACP; clone 9C5, MAB96, Merck Millipore, Helleurup, Denmark) or rabbit anti-Cat K antibodies, or single immunostained with goat anti-osteopontin antibodies (BAF 1433, R&D Systems, Minneapolis, MN, USA). The respective primary antibodies were labeled with alkaline phosphatase/horseradish peroxidase–conjugated anti-mouse/rabbit IgG polymers (BrightVision, Immunologic, Duiven, Holland) or alkaline phosphatase–conjugated streptavidin (Jackson ImmunoVision, Duiven, Holland), visualized with Deep Space Black (Biocare Medical, Concord, CA, USA) and Liquid Permanent Red (DAKO, Glostrup, Denmark), and counterstained with Mayer’s hematoxylin.

**Combined in situ hybridization and immunohistochemistry**

Some of the consecutive sections were in situ hybridized using a modified version of the RNAScope 2.0 high-definition procedure (310035, Advanced Cell Diagnostics [ACD], Hayward, CA, USA) for the mRNA expression of alkaline phosphatase (ALP; probe-Hs-ALPL, 431111, ACD), Runt-related transcription factor 2 (Runx2; probe-Hs-Runx2, 440071, ACD), and collagen type III (Col13; probe-Hs-Col3A1, 549431, ACD). More precisely, the sections were pretreated in pretreatment 1 for 10 minutes at room temperature and pretreatment 2 for 15 minutes at 90°C, whereas pretreatment 3 was replaced with a peptic pretreatment (50% diluted) from the Histology FISH accessory kit (KS799, DAKO). The hybridization and amplification was conducted according to the instructions provided by the manufacturer. The subsequent DAB staining was replaced.
with an additional amplification step using digoxigenin (DIG)-labeled tyramide (diluted 1:500) from the tyramide signal amplification (TSA) plus DIG Reagent kit (NEL748001KT, PerkinElmer, Skovlunde, Denmark) for 5 minutes at room temperature, followed by a labeling with alkaline phosphatase-conjugated sheep anti-DIG FAB fragments (diluted 1:1000) (11093274910, Roche, Basel, Switzerland) and visualized with Liquid Permanent Red (DAKO). Subsequently, the sections were immunostained for TRACP as described above and counterstained with Mayer’s hematoxylin.

### Microscopic analysis and micrographs

Consecutive sections of each selected Haversian remodeling site were immunostained, in situ hybridized, and Masson Trichrome stained. These stainings involved markers of osteoblast lineage cells (CD56, Coll3, ALP, or Runx2), of osteoclasts (TRACP or CatK), and of the cement line (osteopontin). The combined information of these stainings was first used to identify the regions of interest within the cutting cone and the initial part of the closing cone (see Results). These different regions were marked on a printed map. All these markings were validated by a second observer. These markings also provided the observer with a detailed 3D overview, making it possible to measure the length of the cutting cone and its diameter at different levels. Next, the above stainings were used to identify the nuclei profiles of the osteoblast lineage cells as well as the osteoclast profiles within each region of interest. These profiles were marked on the maps, and these markings were also validated by a second observer. Careful establishment of these maps was a key for an appropriate evaluation of quantitative parameters. The number of nuclei profiles of osteoblast lineage cells per bone perimeter and the extent of osteoclast surfaces were assessed in each region of interest by using the OsteoMeasure software (OsteoMetrics, Decatur, GA, USA). The terminology, symbols, and units are in accordance with the ASBMR histomorphometry nomenclature committee.

### 3D reconstruction

The 3D reconstruction was based on a stack of images obtained from 30 consecutive sections of selected Haversian canals with remodeling activity. The osteoclasts, the osteoid surface, and the bone matrix were carefully marked within each of the 30 aligned images in the Amira 4.0 software (Mercury Computer Systems, Mérignac, France), which then could convert these markings into a 3D reconstruction as previously described.

### Statistical analysis

The statistical significance of the differences between the parameters measured in the defined zones of the intracortical remodeling events was calculated using a repeated measures one-way ANOVA and Tukey’s post tests. The statistical significance of the difference between the first and second half of the RvRs phase was calculated using a paired t test. The gradual changes in the successive quartiles of the RvRs phase were calculated using Pearson’s correlation (r). The statistical significance of the difference between assessments at level 1 and 2 of the cutting cone was calculated using a Wilcoxon matched-pairs signed-rank test. The relationship between the RvRs length and other parameters was calculated using a Spearman’s rank correlation.

### Results

Where are the osteoclasts and reversal/osteoprogenitor cells positioned in longitudinal sections of the cutting cone?

TRACP or CatK staining of longitudinal sections of Haversian remodeling sites revealed tightly packed osteoclasts positioned at the tip of the tunnel they are generating (Fig. 1 and Fig. 2A), as typically shown in the literature through conventional histological staining. As expected, mononucleated cells positive for osteoblast lineage cell markers (but not for TRACP or CatK) colonize the newly generated walls of the tunnel as soon as the osteoclast-front moves away (Fig. 1F, G, I, J, L, M). These markers included Runx2, ALP, and Coll3. These mononucleated cells appeared to have the same properties as the reversal cells in adult human cancellous bone and which were recently characterized as osteoprogenitors. They are detected on the eroded surface up to the point where they appear as mature osteoblasts starting bone formation (Fig. 1F–N). This eroded surface is clearly visualized through osteopontin immunoreactivity (Fig. 2A) and corresponds to the so-called cement line upon which new bone is deposited (Fig. 2A).

Very importantly, the TRACP and CatK stainings revealed that osteoclasts are not only present at the tip of the tunnel but are also sparsely distributed amongst the reversal cells on the walls of the tunnel (Fig. 1, 2A). This presence clearly indicates that new resorption events may occur after reversal cells have colonized the eroded surface. However, osteoclasts are never detected at bone formation sites, thereby indicating that resorption events only last until bone formation has started. We defined the osteoclasts at the tip of the tunnel as primary osteoclasts, and those interrupting reversal episodes as secondary osteoclasts. The eroded surface subjected to these alternations of reversal and secondary resorption episodes was accordingly called “reversal-resorption” surface and abbreviated RvR as inspired from the standardized nomenclature. To get a 3D impression of the secondary versus primary resorption sites, we made serial sections and 3D reconstructions (Fig. 1D, E). These reconstructions support the hypothesis that the primary osteoclasts initiating the remodeling cycle mainly contribute to forward resorption along the axis of the tunnel, whereas the secondary osteoclasts contribute to widening of the tunnel. Twenty-six different canals were similarly analyzed in stacks of adjacent sections and all showed the existence of secondary osteoclasts all over the wall in addition to primary osteoclasts at the tip. We conclude that resorptive activity during a remodeling cycle does not proceed as one single continuous step restricted to the initial period of the remodeling cycle, as most often presented, but as multiple steps, where the first one at the tip of the cutting cone mainly contributes to its elongation and is defined here as initial resorption (exerted mainly longitudinally) and where the next resorption steps at the RvRs zone collectively contribute to widening of the cutting cone and are defined here as secondary/radial resorption.

What are the changes in the prevalence of reversal/osteoprogenitor cells and osteoclasts along the cutting cone?

Next, we investigated whether the prevalence of reversal cells and osteoclasts changes along the RvRs. Adjacent sections were made along the longitudinal axis of BMUs. The RvRs surfaces were identified and were divided into four quarters as
Fig. 1. The positions of osteoclasts and reversal/osteoprogenitor cells in longitudinal sections of cutting cones. (A–C) Three adjacent longitudinal sections of a cutting cone in the fibula of a 65-year-old man were respectively Masson’s trichrome stained (A) and immunostained for the osteoclastic markers TRAcP (B) and cathepsin K (CatK) (C). The latter two markers reveal tightly packed osteoclasts at the tip (“1st osteoclasts,” orange arrowheads) and sparsely distributed osteoclasts on the eroded walls (ES) of the cutting cone (“2nd osteoclasts,” yellow arrowheads), up to the starting point of osteoid deposition (OS). (D, E). Two views of the 3D organization of osteoclasts (red) within a cutting cone in the femur of a 12-year-old boy. The 3D reconstruction was generated by aligning micrographs from 30 serial sections immunostained for CatK (red). The pictures show the cutting cone opened along its longitudinal axis, up to where bone formation starts (OS, blue). They reveal tightly packed osteoclasts at the tip (“1st OCs”) and a sparse distribution of osteoclasts on the wall (“2nd OCs”). This distribution indicates initial and later resorption periods, playing a role in respectively elongating and widening the cutting cone. (F–N) Position of reversal/osteoprogenitor cells respective to osteoclasts. Three serial sections of a cutting cone in the femur of a 10-year-old girl were in situ hybridized for the mRNA of the osteoblastic markers collagen type 3 (Coll3 in F–H), alkaline phosphatase (ALP in I–K), or Runx2 (L–N) (red) and subsequently immunostained for TRAcP (black). Note that reversal/osteoprogenitor cells (green arrows) appear on the eroded wall (ES) immediately after the initial resorption period (orange arrowheads) and are present together with scattered secondary osteoclasts (yellow arrowheads) all along the cutting cone up to the onset of osteoid deposition (OS). The latter intermixed cell population points to the existence of a mixed “reversal-resorption” phase. Scale bars = (A–C, F, I, L) 50 μm; (G, H, J, K, M, N) 20 μm.
Fig. 2. Changes in the prevalence of osteoclasts and reversal/osteoprogenitor cells along the cutting cone. Serial longitudinal sections of 17 cutting cones were made as close as possible to their central axis and stained for an osteoclast and an osteoblast lineage cell (CD56, Coll3, ALP, Runx2) or cement line marker. (A) The pictures show an example of two of the serial sections of a cutting cone, immunostained for the osteoclastic marker CatK (black) and the osteoblast lineage cell marker CD56 (upper) or the cement line marker osteopontin (lower; red), respectively. Note the 1st osteoclasts (orange arrowheads), 2nd osteoclasts (yellow arrowheads), and the cement line (white arrowheads) that becomes embedded in the matrix where bone formation has started (F). Six distinct zones of the remodeling surface were defined: the initial resorption surface (I.Rs, 1st osteoclasts), the RvRs surface (reversal cells and 2nd osteoclasts) divided into four equal quarters (Q1–Q4), and the early osteoid surface over a perimeter equal to one quarter of the RvRs (F). Scale bars = 50 μm. (B, C) The densities of osteoblast lineage cells (number of nuclei per mm bone perimeter [N.Nc.Pf/B.Pm]) (B) and the extent of osteoclast surfaces (Oc.S/BS) (C) were estimated in each of these zones on at least two non-neighboring sections of each of the 17 cutting cones. The measurements in the respective zones of each cutting cone are shown as a dot. For each zone, the mean of the measurements obtained in the different cutting cones is shown as a short horizontal line. (B) Note that the densities of the osteoblast lineage cells gradually increase along the cutting cone and reach a value of at least 39 (dotted line) at the onset of osteoid deposition. The overall change over the four quarters of the RvRs phase was analyzed by using the Pearson correlation (r_p = 0.70, p < 0.0001). The red line represents the best-fitted line. The differences between the zones were analyzed using one-way ANOVA followed by Tukey’s multiple comparison tests: dashed line p < 0.01; plain line p < 0.001. (C) Note the permanence of osteoclasts along the RvRs surface, although they are less abundant in the second half of the RvRs surface, as analyzed by using a paired t test (p = 0.022).

shown in Fig. 2A. Osteoblast lineage cell densities and osteoclast surface per bone perimeter were assessed in each of these quarters as well as on the early osteoid surface (measured over a perimeter equal to one quarter of the RvRs perimeter). Fig. 2B shows that the average cell density gradually grows from the first to the fourth quarter and even increases further when osteoid deposition has started. This indicates a continuous cell recruitment during the RvRs phase and during the early osteoid deposition. The cell densities reached at this latter stage were quite variable amongst different BMUs but never below 39 nuclear profiles per mm perimeter, supporting the concept of a threshold cell density for matrix deposition. Interestingly, this concept is also supported by the in vitro bone nodule bone formation assay showing higher cell densities at the collagen deposition sites (Supplemental Fig. S1), although to our knowledge these densities were never quantified.\(^{28,29}\)

The extent of osteoclast surfaces appears to vary less than reversal cell densities amongst the four quarters of the RvRs surfaces (Fig. 2C). Osteoclasts were still present up to the fourth quarter and sometimes up to very close to the point where osteoid deposition started (Fig. 1F and Fig. 2A). Still, the osteoclast surfaces were significantly lower in the last two quarters compared with the first two. Once bone formation started, osteoclasts were no longer found (Fig. 2C). We conclude that the progression of the RvRs phase goes
along with increased colonization of the bone surface with reversal cells up to a threshold and with a permanent presence of osteoclasts sparsely distributed amongst the reversal cells.

Assessment of the levels of bone remodeled through secondary versus initial resorption

Next, we evaluated the amount of bone remodeled through secondary and initial resorption and their relative contribution to overall bone resorption in individual bone remodeling units. In 3D, the total volume of bone resorbed by a remodeling unit has the shape of a cylinder whose cross-section area equals that of the osteon—this is the cross-section area of the canal at the level where osteoid deposition starts ([14,15], Fig. 3A). Similarly, the volume of bone removed by initial resorption can be approximated to a cylinder whose cross-section area equals that of the canal at the level where initial resorption ends—which is where the first reversal cell colonizes the eroded surface (Fig. 3A). To obtain these measurements, adjacent sections parallel to the respective longitudinal axes of 26 canals were obtained. For each canal, the section passing at the level of the central axis of the canal was selected. On each of these central sections, the beginning and end of the RvRs surface were determined, respectively, by the position of the first reversal cell and the initiation of osteoid deposition. The diameter of the canal was measured at these two positions and taken as the diameters reached by “initial” (level 1) and “initial plus secondary” (level 2) resorption, respectively (Fig. 3A). These measurements were plotted in Fig. 3B and all canals showed a larger diameter at level 2 compared with level 1, thus clearly indicating a widening of the canal due to secondary resorption. To evaluate the contribution of secondary resorption to overall resorption, we used these diameters to calculate the cross area of the canal reached at the end of the initial resorption period and at the end of the RvRs period (Fig. 3C) and to calculate the proportion of bone removal due to secondary resorption. As shown in Fig. 3D, the

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**Fig. 3.** Assessment of the levels of bone resorbed during the initial resorption phase and the RvRs phase. (A) The scheme illustrates a cutting cone sectioned along its central axis. It shows that the resorption occurring during the initial resorption period (I.Rs) and the RvRs phase can roughly be evaluated by measuring the cutting cone diameter (i) at the level where the reversal cell recruitment onto the eroded surface starts (level 1) and (ii) where the osteoid deposition is initiated (level 2) (for more details, see text). (B, C) In all 26 investigated cutting cones, the diameters (B) and the corresponding cross areas (C) at level 2 are larger than those at level 1, demonstrating resorption during the RvRs phase (Wilcoxon matched-pairs signed-rank test: ***p < 0.0001). (D) The values found in panel C were used to calculate which percentage of resorption was generated during the I.Rs (red bars) and during the RvRs period (green bars), respectively, in the 26 investigated cutting cones. The red dashed line represents the median contribution. Note that the contribution of the RvRs phase to overall resorption in the respective cutting cones was at least 48%.
contribution of secondary resorption varies strongly depending on the BMUs but is always above 48% and up to 95% with an average of 83%. Thus secondary/radial resorption is the main contributor to the overall resorption occurring in a BMU.

Relation between variations of the length of the RvRs zone, widening of the cutting cone during the RvRs phase, permanence of secondary osteoclasts, and osteoblast lineage cell density

We noticed a widespread variation in the absolute length of the RvRs zone amongst the different BMUs, thus indicating great variations in the time lag for starting bone formation after the RvRs zone (fivefold variation between the smallest and the largest RvRs length) (Fig. 4). We hypothesized that longer RvRs zones would lead to longer exposures of the surfaces to secondary resorption and thus to a greater widening of the canal. We, therefore, considered the initial canal width (level 1 diameter in Fig. 3A) and its maximal width (level 2 diameter in Fig. 3A, reflecting the future osteon diameter). When relating either the latter width or the actual width (level 2 minus level 1 diameter) with the length of the RvRs zone, we found significant correlations, thereby supporting the hypothesis that the widening of the canal increases commensurately with the length of the RvRs zone (Fig. 4A). This hypothesis implies that osteoclasts are present until the end even when the RvRs zones are longer. This proved to be the case because even the canals with the longest RvRs zones showed osteoclasts in the last quarter of the RvRs zone (data not shown). This indicates the existence of a mechanism allowing the permanence of osteoclasts along the whole RvRs zone even when the RvRs zone is long. Furthermore, Fig. 4B shows that the osteoblast density at the onset of the bone formation step is independent of the variable length of the RvRs surface in different BMUs. Because the latter variations reflect a variable lag time for the onset of bone formation after the initial resorption period, the data of Fig. 4B also mean that the threshold density required for bone formation (Fig. 2A) is reached after variable times. This, therefore, points to variations in the rate of reversal/osteoprogenitor cell recruitment amongst different BMUs. Taken together, these observations indicate that the rate of reversal/osteoprogenitor cell recruitment influences the length of the RvRs zone and the persistence of bone removal through secondary resorption. According to these data, the slower the recruitment of reversal/osteoprogenitor cells, the later the initiation of bone formation, the longer the length of the RvRs zone, the higher the temporary bone deficit created during the bone remodeling cycle, and the wider the diameter of the osteon to be formed.

Discussion

The picture of a bone remodeling unit is commonly obtained by assembling in a logical order, fragmentary views of different BMUs cross-sectioned at random levels. The originality of the present study is to capture longitudinally single BMUs with specific cell markers. This enables one to obtain the real picture of the whole range of cellular events occurring between the initial resorption episode up to the initiation of bone deposition as a functional continuum. This “one-shot” view reveals (i) that bone matrix is subjected to several resorption episodes, separated by reversal periods during which increasing numbers of reversal/osteoprogenitor cells are recruited—instead of one period of pure resorption followed by one period of pure reversal, as commonly depicted, and (ii) that once a threshold reversal/osteoprogenitor cell density is reached, bone formation is initiated and resorption is switched off (Fig. 5). It leads to a model where the rate of reversal/osteoprogenitor cell recruitment puts the brakes on the extent of resorption (Fig. 5).

The cutting cone integrating resorption and osteoprogenitor recruitment

There have been only a few histological reports on longitudinal sections of Haversian remodeling sites and even less so in humans. Of note, the sections used in these early studies were not subjected to stainings for specific osteoclastic and osteoblastic markers. The space created by remodeling was merely described as a tunnel first of increasing diameter.
showing eroded surface, ascribed accordingly to resorption and called the cutting cone, and thereafter of decreasing diameter and with osteoid surface, ascribed to formation and called the closing cone.\(^{14,15}\) The transition between the cutting and the closing cones was vaguely proposed as the narrow “reversal” space where osteoprogenitors are recruited on the eroded surface.\(^ {14,15,23}\) This rudimentary model has been inspiring many cortical bone studies\(^ {30,32–34}\) and has been the basis of the BMU concept.\(^ 9\) However, the specific cell stainings of the present study show both osteoprogenitors and scattered osteoclasts all over the walls of the cutting cone, thereby revealing resorption and osteoprogenitor recruitment as an intimately integrated process (Fig. 5A). This intricate configuration opens the way for understanding the complex list of factors that were recently reported to control osteoclast-osteoblast interactions.\(^ {1,2}\)

**Osteoprogenitor recruitment**

Our study investigates for the first time reversal/osteoprogenitor cell recruitment as a continuum along the BMU and thereby highlights several important properties: (i) Reversal/osteoprogenitor cells appear immediately after the initial resorption episode, as could be expected from observations in cancellous bone.\(^ {3,4,6,25,26}\) (ii) The density of these cells is increasing along the cutting cone. (iii) Osteoid is detected only above a critical cell density, indicating that a threshold density has to be reached for initiating osteoid deposition. This need was already suggested by osteoblast density measurements in cancellous bone\(^ {20}\) and has led us to propose that poor osteoprogenitor recruitment on reversal surfaces compromises initiation of bone formation.\(^ {3–5,35}\) Also supporting this threshold concept are the incidental pictures taken during bone nodule formation assays, which show that cell densities are increased when bone matrix deposition occurs, but to our knowledge, these were never quantified.\(^ {28,29}\) The reason for a threshold cell density remains to be investigated but may relate to cell shape–regulated gene expression.\(^ {36}\) The acquisition of a cuboidal shape by osteoblast lineage cells is commonly associated with collagen secretion\(^ {37}\) and demands enough cells in the cell layer lining the bone surface. (iv) Finally, our study highlights that the threshold cell density for starting bone formation is reached over very variable distances of eroded surface, depending on the BMU. Of note, this variability suggests differences in the rate of reversal/osteoprogenitor cell recruitment relative to the rate of cutting cone elongation (Fig. 5B).

**Fig. 5.** Graphical integration of the observations into a model. (A) The present observations demonstrate that a remodeling cycle shows multiple resorption periods and not just one occurring at its start. These resorption periods alternate with recruitment of reversal/osteoprogenitor cells onto the eroded surface, thus revealing the existence of a mixed RvRs phase. Once a threshold cell density of reversal/osteoprogenitor cells is reached, bone formation is initiated and no more resorption periods occur. (B) This threshold is reached after variable lengths of RvRs surface, depending on the BMU, thus indicating a variable rate of reversal/osteoprogenitor recruitment amongst different BMUs. Overall, these observations reveal that the rate of reversal/osteoprogenitor recruitment may affect the extent of resorption. For more details, see text.
Longitudinal versus radial resorption controlled by osteoprogenitor recruitment rate

Several authors have discussed the relation between the length of the cutting cone and its maximal diameter (i.e., the future osteon diameter), which reflect, respectively, longitudinal and radial resorption.\(^{(15,22,38,39)}\) The mechanism behind this relation is clarified by the present study. The primary osteoclasts packed at the tip of the Haversian canals appear to initiate resorption and exert their resorption mainly along the longitudinal axis of the canals, whereas the secondary osteoclasts scattered over the eroded walls of the canals exert additional/secondary resorption episodes that collectively prevail in a radial direction. This radial orientation is clearly indicated by the radial displacement of the cement line and by the increasing diameter of the canal after the initial resorption has stopped. This increase lasts until the point where bone formation is initiated, as osteoid then protects the bone surface against further osteoclast resorption.\(^{(40,41)}\) This means that the exposure time of the walls of the cutting cone to secondary/radial resorption is directly proportional to the rate of elongation of the cutting cone and inversely proportional to the rate of osteoprogenitor recruitment.

Interestingly, the length of the cutting cone is determined by exactly these two same factors, thereby explaining the correlation between the length and the maximal diameter of the cutting cone (Fig. 5B). The secondary/radial resorption proceeds at a slower rate because it results from scattered osteoclasts. Furthermore, the extent of osteoclast surfaces is decreasing as the RvRs progresses (Fig. 2C), which may explain the ellipsoidal shape of the cutting cone. Still, our data show that this secondary/radial resorption contributes the most to bone matrix removal from a quantitative point of view. It remains to be investigated whether the scattered osteoclasts exerting radial resorption are new osteoclasts continuously recruited from the lumen or osteoclasts that earlier exerted longitudinal resorption at the tip of the canal, which would imply continuous renewal of the osteoclasts at the tip.

It is worth noting that although longitudinal and radial resorption may be similarly regulated by osteocyte signals,\(^{(42)}\) only radial resorption (the main contributor to overall resorption) can be affected by interactions with reversal/osteoprogenitor cells, cement line epitopes,\(^{(4)}\) and importantly, the rate of reversal/osteoprogenitor cell recruitment. The sooner bone formation is initiated, the faster secondary bone resorption will stop and as a result the lower the temporary bone porosity created during the bone remodeling cycle and the narrower the diameter of the osteon to be formed. This mechanism may contribute to the “antiresorptive” response to treatments aimed at stimulating bone formation, such as anti-sclerostin drugs,\(^{(43,44)}\) and inversely, one may also think of its possible contribution to generation of porosity in situations where osteoprogenitor recruitment is deficient, such as aging.\(^{(16,38)}\)

Relevance to cancellous bone

Because it has been proposed that cancellous bone BMUs can be regarded as hemi-osteons,\(^{(14)}\) one may wonder whether the present findings apply to cancellous bone remodeling. Osteoclasts responsible for initial resorption at the tip of the canal, thereby elongating it, would be homologous to osteoclasts at the edge of the remodeling site of cancellous bone, which travel across the quiescent surface, thereby extending the eroded surface.\(^{(14,38,39)}\) Similarly, the osteoclasts responsible for secondary resorption on the walls of the canal, thereby widening it, would be homologous to the cancellous bone osteoclasts that re-erode already eroded surfaces, making excavations deeper. In accordance with this view, the differences in osteoclast density, respectively on the wall and at the tip of the cutting cones, correspond with the differences in osteoclast density at respectively shallower and deeper depths at cancellous bone erosion sites.\(^{(10)}\) Just as the rate of recruitment of osteoprogenitors appears to determine both the time lag for initiation of bone formation and the degree of widening of the canal through secondary resorption, the rate of recruitment of osteoprogenitors would determine both the time lag for initiation of bone formation and the final erosion depth in cancellous bone. Interestingly in this respect, cancellous bone in postmenopausal osteoporosis, a situation where local osteoprogenitors are deficient,\(^{(3,35)}\) shows an increased time lag for initiation of bone formation\(^{(3,45,46)}\) and increased excavation depths when bone formation fails to start (i.e., aborted remodeling cycles).\(^{(3)}\) In contrast, PTH, which favors the availability of osteoprogenitors,\(^{(47,48)}\) reduces the time lag for initiation of bone formation\(^{(3,11,46)}\) and decreases excavation depths in cancellous bone.\(^{(11)}\) The relevance of the present observations to cancellous bone is also supported by basic observations, such as the frequent presence of reversal/osteoprogenitor cells incidentally shown at both sides of osteoclasts on eroded surfaces,\(^{(3,6)}\) thereby indicating an interruption of the reversal phase by resorption episodes. Intimate interactions between osteoclasts and reversal cells were also reported in cancellous bone.\(^{(3,6,25,26)}\) Finally, initiation of bone formation in cancellous bone also appears to require a critical cell density, as mentioned above.\(^{(3,5,20,35)}\)

Conclusion

The present study reveals basic modalities of the bone remodeling cycle that are commonly overlooked (Fig. 5). One may distinguish an initial resorption event that is responsible for extending erosion in a given direction (elongating the Haversian canals) and secondary resorption events that are responsible for extending erosion in the other directions (which make the Haversian canals wider or may make resorption deeper in cancellous bone). The latter events contribute the most to overall resorption and interrupt the reversal period. This integrated resorption and reversal phase (RvRs) allows multiple osteoclast-osteoblast lineage cells interactions. This RvRs phase goes along with a gradual increase in the density of osteoprogenitor cells. When a threshold is reached, bone formation starts and secondary resorption events stop. Jointly, these observations point to a model where the rate of osteoprogenitor recruitment conditions the extent of bone degradation. This knowledge was obtained on Haversian canals with a symmetrical organization of the remodeling events. It should help in also addressing what goes wrong in more complex situations, which may, for example, result in gain in porosity.\(^{(16–18)}\) Furthermore, the present work highlights the interest of histological approaches designed to investigate the spatiotemporal mechanism of a biological process and which can be directly performed on human material.

Disclosures

All authors state that they have no conflicts of interest.
Acknowledgments

We thank Birgit MacDonald, Kaj Rau Laursen, and Jacob Baethelm Olesen for their outstanding technical assistance and the Region of Southern Denmark (grant no. 13/7133) and The Velux Foundation (VELUX34368) for financial support.

Authors’ roles: The study was designed by J-MD and TLA and conducted by NEL assisted by TLA. Haoversian BMUs were collected by EMH, SH, and GETE. The data were analyzed and interpreted by NEL, TLA, KS, and J-MD. The manuscript and the illustrations were drafted by J-MD and TLA, respectively. All authors approved the final version of the manuscript. TLA takes responsibility for the integrity of the data analysis.

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