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Perspective Article

Hylonome

Osteocyte-osteoclast morphological relationships and the putative role of osteocytes in bone remodeling

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Abstract

An osteocyte lacunae differential count under the light microscope (LM) (1-lacunae with live osteocytes, 2-empty lacunae and lacunae with degenerating osteocytes) was carried out outside the reversal lines of osteonic lamellar bone from various mammals and man to evaluate the possibility of osteocyte survival where osteoclast resorption had occurred. The polarized light microscope (PLM) was used to establish the curvature of bony lamellae outside the convexity of reversal lines: concave lamellae indicate osteocytes reabsorbed on their vascular side where they radiate long vascular dendrites; convex lamellae indicate bone resorption on the osteocyte mineral side, radiating short dendrites. In all samples it was found that: a) about 60% of osteocytes outside the reversal lines were live; b) the percentage of alive osteocytes close to reversal lines is higher when they are attacked on their mineral side. The present data support our view that surviving osteocytes, particularly those attacked from their mineral side, might intervene in the final phase of bone resorption (osteoclast inhibition?). The fact that under the transmission electron microscope (TEM) intercellular contacts were never observed between osteocytes and osteoclasts indicates that if a modulation should occur between these two cellular types it could take place by a paracrine route only. The putative role of the cells of the osteogenic system, particularly osteocytes, in the bone remodeling cycle is also discussed.

Keywords: Osteocyte, Osteoclast, Osteogenic System, Reversal Line, Bone Remodeling

Introduction

Notwithstanding the large number of *in vitro* and *in vivo* studies carried out during recent years on the function of bone cells, the precise mechanism by which they interact during the bone remodeling process is far from clear.

In a series of morphofunctional investigations carried out in our laboratory, we demonstrated that osteocyte cytoplasmic arborization is highly asymmetrical, with vascular dendrites (i.e. those radiating towards the bone vascular surfaces) far longer than the opposite mineral ones[1-3], and that the overall osteogenic cell system (stromal cells, osteoblasts or bone lining cells, osteocytes) forms a continuous cytoplasmic network having the characteristic of a functional syncytium, because of the gap junctions it contains. We also provided evidence to support our view that osteogenic cell activity may be modulated not only by *volume transmission*, as generally admitted, but also by *wiring transmission*, namely in a neuronal-like manner[4-8].

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It has been clearly shown that the cells of the osteogenic lineage, besides directing bone formation in response to both mechanical and metabolic signals, are also involved in bone resorption. In fact, the formation of osteoclasts, and possibly also their activation is dependent upon contact between haemopoietic precursors and cells of the osteoblast lineage. The latter promote osteoclast formation from precursors through the production of M-CSF and RANKL[9].

While we should now accept that the osteogenic cell system, in particular its constituent elements during the bone resting phase (osteocytes, bone lining cells, stromal cells, namely the *Basic Bone Cellular System* according to Marotti)[6], occupies a central position not only in bone formation but also in activating bone resorption, whether it also intervenes in arresting osteoclast activity remains to be clarified. Indeed, several systemic and local factors have been claimed to intervene in arresting bone resorption, but the precise cell-mediated mechanism by which this phenomenon occurs is far from being established.

In the present histomorphometric study, which builds on previous preliminary reports[10-11], we start from the premise that, during bone resorption, osteoclasts must necessarily destroy stromal and bone lining cells (unless they retract or

disappear owing to other causes) before reabsorbing the underlying bone matrix, and that the only cells of the osteogenic system that can survive osteoclast disruption are, if any, the osteocytes. Accordingly, we evaluate osteocyte viability just outside the reversal lines of secondary osteons, namely in sites where osteocytes were sure to have been located close to osteoclasts, in order to investigate the putative existence of osteocyte-osteoclast modulation. The ultrastructure of osteocytes along resorption cavities is also analyzed.

Materials and methods

Histomorphometry

Analyses were performed on cross-sections (3mm thick) of compact lamellar bone at the mid-diaphyseal level of tibiae in adult animals (3 pigs, 3 sheep) and in biopsies taken from the femoral neck of 6 arthrosic male patients, aged between 60 and 65, subjected to hip prosthesis. Soon after removal, the entire sections were divided into quadrants or sectors, according to their size, and fixed in 4% buffered paraformaldehyde (phosphate buffer 0.13mol/L, pH 7.2) for 3 hours, dehydrated in increasing graded ethanol and embedded in methylmethacrylate (MMA). Thin undecalcified sections (5μm thick), obtained with a bone microtome (Autocut 1150 Reichert Jung) and stained with 0.05% Cresyl Violet in 0.1 sodium, were analyzed under transmitted ordinary light by means of a Zeiss Photomicroscope (LM) equipped with an Image Analyzer (Vidas Zeiss) to perform the following osteocyte lacunae differential count: 1) osteocyte lacunae containing live cells, i.e. osteocytes showing ill-defined and well-preserved nuclei; 2) empty lacunae and lacunae containing degenerating osteocytes. All lacunae displayed the typical almond-like shape of those in lamellar bone. These parameters were evaluated in each quadrant or sector, making up 5 entire sections from each subject, both in the whole section surface and in the 30μm thick bands outside the reversal lines of secondary Haversian systems with a lamellar structure (Fig. 1). At least 150 osteocyte lacunae, located in the 30μm bands, were counted in each section; all lacunae were taken into account independently of the orientation (tangential, perpendicular or oblique) of their elliptical outline with respect to the convexity of the reversal lines. The value of 30μm was selected because it roughly corresponds to the mean distance between adjacent osteocyte cell bodies (personal unpublished data), and thus presumably also to the approximate length of their vascular dendrites (see above). In this connection, we attempted to discover whether a difference exists in osteocyte behaviour depending on whether its vascular or mineral dendrites are first attacked by the osteoclast. Transmitted polarized light (PLM) was used because it allows the curvature of bony lamellae to be established with respect to the convexity of reversal lines. Concave lamellae outside convex reversal lines indicate that bone resorption had occurred along the osteocyte vascular side, from which the long dendrites radiate; convex lamellae indicate the opposite, i.e. bone resorption on the osteocyte mineral side (Fig. 2). The 30μm bands outside the reversal lines were divided into three concentric zones (each 10μm thick) (Fig. 3), and osteocyte lacunae differential count was performed in each 10μm zone. Only osteocyte lacunae whose elliptical outlines were tangential to the convexity of the reversal lines were taken into account; perpendicular and oblique lacunae were discarded because of pertaining to osteocytes attacked neither on the vascular side nor on the mineral one. Statistical analyses were performed with *One way ANOVA test* and *Tukey multiple comparison Test.*

Figure 1. Cross-sectioned human Haversian system under transmitted semi-polarized ordinary light (x460). The arrows point to two osteocyte lacunae close to the convex external side of the reversal line.

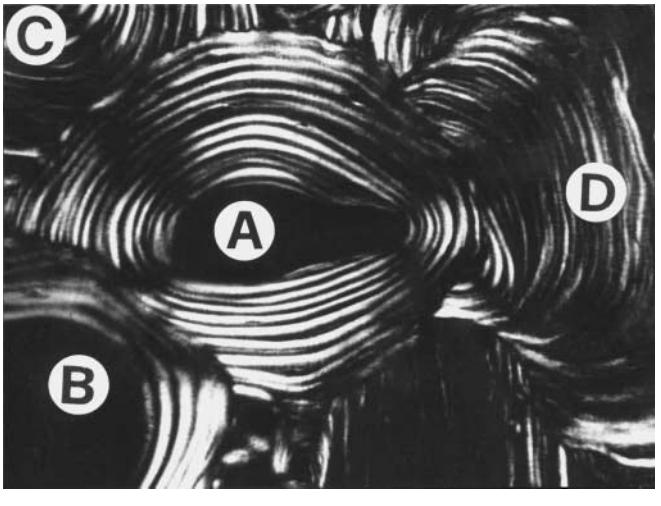

Figure 2. Cross-sectioned human cortical bone under transmitted polarized light (x250). On the basis of the convexity of the reversal lines, it can be inferred that A osteon was laid down before B osteon and after C and D osteons. Consequently, osteocytes in A and C osteons were attacked by osteoclasts on their mineral side, whereas those in D osteon on their vascular side.

Transmission Electron Microscopy (TEM)

Ultrastructural analyses were performed on cross-sections (2mm thick) from growing animals (pig, sheep) at the mid-diaphyseal levels where bone remodeling processes occur at a higher rate. Samples were fixed in 4% buffered para-formaldehyde (phosphate buffer 0.13mol/L, pH 7.2) for 2 hours, postfixed in 1% osmium tetroxide (phosphate buffer 0.13mol/L, pH 7.2) for 2 hours, dehydrated in increasing graded ethanol and embedded in epoxy resin (Durcupan ACM). Some samples were decalcified with 2.5% EDTA in phosphate buffer before postfixation. All specimens were sectioned with a diamond knife (Ultracut-Reichert Microtome) in order to obtain ultrathin sections (70-80nm thick). The sections were mounted on Formvar- and carbon-coated copper grids, stained with 1% uranyl acetate and lead citrate, and photographed under a Zeiss EM 109 transmission electron microscope.

Results

Histomorphometry

The results of the osteocyte lacunae differential count, performed in the whole sections and 30 μm thick bands outside the reversal lines, are reported in Figure 4. This computation includes all osteocyte lacunae independently of their orientation with respect to the reversal lines. It appears that in all animal species, including man, no differences in the percentage of live osteocytes exist between the whole sections and the 30μm bands outside the reversal lines (Fig. 4). Neither were any differences found among the various animal species examined, including man.

Table 1 shows the percentage values of live osteocytes in the three 10μm zones into which 30μm bands were divided. In order to establish the vascular or mineral sides from which osteocytes are attacked by osteoclasts, this count was restricted to osteocyte lacunae whose elliptical outlines were tangential to the convexity of the reversal lines. Therefore the data in Table 1 are not comparable with those in Figure 4. From A to C zone (i.e. from the reversal line outwards) the percentage value of live osteocytes increases when the reversal lines face the osteocyte vascular side and does not change significantly when the reversal lines face the osteocyte mineral side. It is to be noted that in A zone, close to the reversal lines, the percentage of live osteocytes attacked by osteoclasts on their vascular side is about half that of osteocytes reabsorbed on their mineral side.

	Vascular side	Mineral side	*Tukey Test*
A (0-10μm)	25.8±0.95	50.7±0.44	*p<0.5*
B (10-20μm)	34.8±0.77	49.3±0.83	*p<0.5*
C (20-30μm)	39.4±0.21	51.2±0.81	*p<0.5*
Anova Test	*p≤0.001*	*p≤0.151*	

Table 1. Percentage values of live osteocytes (± Standard Error) evaluated in all samples from the three zones (A, B, C) into which the 30 microns bands outside the reversal lines were divided. Vascular side and mineral side indicate the side of osteocyte facing the reversal line, i.e. the direction of osteoclast resorption. *One way ANOVA test* shows that, among ABC zones, mean values significantly differ in vascular side, whereas they do not in mineral side. *Tukey multiple comparison test* shows that a significant difference exists between the mean values of vascular and mineral sides in each zone.

Transmission electron microscopy (TEM)

Ultrastructural observations of resorption cavities show that the majority of osteocyte lacunae either appear to be empty or contain osteocytes at different degrees of degeneration

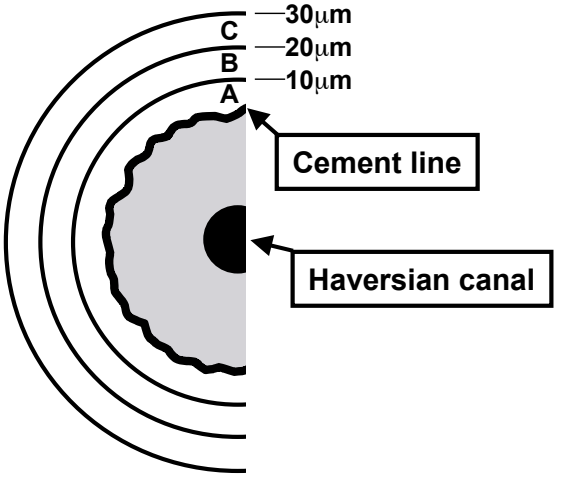

Figure 3. Schematic drawing showing the three 10μm thick concentric zones (A, B, C) into which the 30μm bands outside the reversal lines were divided.

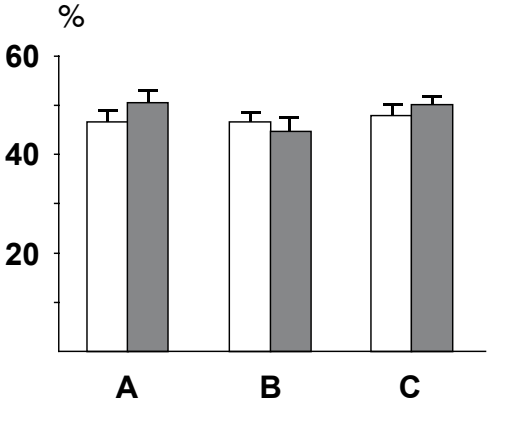

Figure 4. Mean percentage values of live osteocytes (± Standard Error) evaluated from all samples examined in man (A), pig (B) and sheep (C). Open bars: osteocyte viability in the whole sections. Closed bars: osteocyte viability in the 30μm bands outside the reversal lines. There are no significant differences in the percentage of live osteocytes neither between the whole sections and the 30μm bands outside the reversal lines, nor between the various animal species examined.

(Fig. 5). However some lacunae contain live osteocytes displaying a well-preserved cell body and dendrites radiating towards the ruffle border of the osteoclasts (Fig. 6). Thick cytoplasmic extrusions of osteoclast clear zone are also observed entering empty osteocyte canaliculi for a short distance. However, neither specialized junctions nor intercellular contacts are found between osteocytes and osteoclasts.

Discussion and conclusions

The first point to consider is whether the method we used to perform osteocyte lacunae differential count under LM is valid. To avoid any possible misinterpretation of osteocyte survival, we consider as alive only those cells displaying an ill-defined and well-preserved nucleus. This fact obviously implies an underestimation of living osteocytes, though their nucleus-to-cytoplasm ratio is so high that the possibility of finding cells not sectioned at the nuclear level is very low. However, since such underestimation applies to all osteocyte differential counts, comparisons of the findings for the various samples examined must be considered valuable.

While the osteocytes we found alive outside the reversal lines were surely alive at the time of osteoclast erosion, the dead osteocytes could have died afterwards. This possibility is partly corroborated by the fact that in no cases were significant differences recorded in the percentage of live osteocytes between the 30μm bands outside the reversal lines and the entire sections. Briefly, it is likely that the number of osteocytes surviving towards the end of osteoclast erosion is higher than that here reported. It is clear from our findings that osteocyte death does not necessarily occur during the final phase of osteoclastic bone resorption.

The existence of a huge amount of dead osteocytes in all samples examined appears to be consistent with previous observations that bone with dead osteocytes does not necessarily undergo remodeling[12-13]. Thus we do not agree with those authors who suggest that osteocyte death represents the main causal factor of bone resorption. However, it is very likely that in some circumstances, such as fatigue damage, damaged or apoptotic osteocytes close to microcracks might trigger and direct osteoclast activity, as suggested by several authors[14-18].

The data reported in Table 1 can be explained on the basis of our previous studies on osteocyte dendrite arborization[1-3]. Since vascular dendrites were found to be incomparably longer than mineral ones, the fact that, in A zone, the percentage of live osteocytes is far lower (about 50% less) when bone resorption occurs on the osteocyte vascular side indicates that the osteocyte is affected by the osteoclast from the time the latter starts to damage its dendrites. For the same reason, the osteocyte attacked on its mineral side (where its dendrites are very short) can survive at a shorter distance from the osteoclast, practically until the latter comes into contact with the osteocyte cellular body (Fig. 7). These findings suggest that the osteocyte attacked on its vascular side does not seem to retract its dendrites as the osteoclast advances, otherwise it would not suffer osteoclast disruption at a greater distance than the osteocyte attacked on its mineral side. They also show that, of the osteocyte population, the candidate most likely to play a role in modulating the final phase of osteoclast resorption is the osteocyte attacked on its mineral side, since, according to our findings, it has a twofold chance of surviving close to osteoclasts.

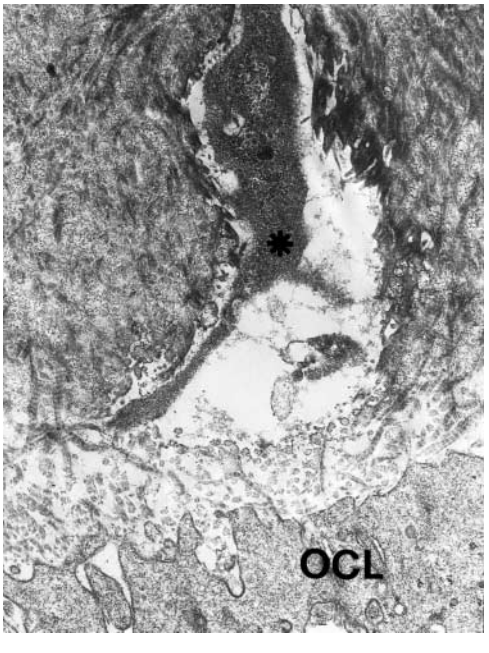

Figure 5. TEM micrograph (x24,000) of a decalcified bone sample showing a degenerating osteocyte (*), with a melted coarse-grained cytoplasm and an undefined plasma membrane, close to the ruffle border of an osteoclast (OCL).

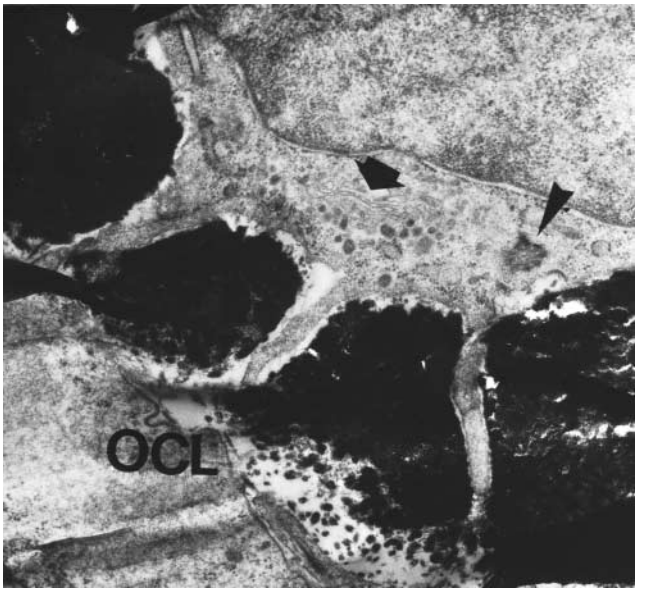

Figure 6. TEM micrograph (x24,000) showing a well-preserved osteocyte. The arrow points to the Golgi apparatus, the arrow-head a centriole. Note three osteocyte dendrites reaching the resorption cavity without coming into contact with the osteoclast (OCL).

An osteocyte-dependent local control of bone remodeling has already been theoretically supported by Mullender and Huiskes[19], but the effect exerted by osteocytes on osteoclasts, osteoblasts and bone lining cells is still a matter of pure speculation. However, thanks to results here reported, showing for the first time that an unexpected high number (about 60%) of osteocytes survive at the end of osteoclast disruption, and on the basis of (a) the *Mechanostat Setpoint Theory and Utah Paradigm*[20-23], (b) the fact that osteocytes have recently been shown to act as mechanosensors[24-26], likely capable of modulating bone resorption and bone formation when the lower (about 50μΣ) and upper (about 2,500μΣ) setpoint values respectively are exceeded[5] (those indicated here are most comonly reported in the literature), and (c) our recent preliminary investigations showing that osteocytes in metatarsal bones of weanling mice, subjected *ex vivo* to axial pulsing loading cycles, increase and maintain steady the ionic current induced by strain inside the lacuno-canalicular microcavities[27], the following hypothetical sequence of events is postulated as occurring during bone resting and remodeling.

Resting Phase. Physiological pulsing mechanical loads keep microstrain values within the physiological window. Under these conditions, the steady ionic current driven by osteocytes[8,27] inhibits, probably by *wiring transmission*, the cells of *Bone Basic Cellular System* (BBCS) outside the bone matrix (bone lining cells and stromal cells), and the bone remains in a resting state. In fact it is hard to believe osteocytes are continuously engaged in sending excitatory signals; this would be a biological nonsense.

Bone Remodeling Cycle. Under unloading (<50μΣ) or when osteocyte sensitivity to strain is increased by setpoint altering agents (PTH, Estrogens, etc.)[5], osteocytes stop producing a steady ionic current and the bone lining cells and/or stromal cells are no longer inhibited and produce osteoclast activating cytokines (RANKL), which induce by *volume transmission* osteoclast differentiation and activation, thus triggering a bone remodeling cycle (*1st Phase-Resorption*) (Fig. 8). During bone resorption, BBCS is destroyed, but the only cells having the possibility of stopping osteoclast activity are the osteocytes. In fact, osteocytes appear to suffer bone disruption passively; however, when the local upper setpoint (2,500μΣ) is exceeded, because of bone strain increment along the wall of the resorption cavity, it is likely that surviving overstrained osteocytes stop osteoclast activity and the reversal phase (*2nd Phase-Reversion*) then starts. The cells of the reversal phase (probably of stromal-fibroblast origin) differentiate into osteoblasts and bone formation occurs (*3rd Phase-Deposition*). During bone deposition, BBCS is progressively rebuilt and osteoblast activity stops when local microstrain values fall again within the physiological window, because the osteocytes in the newly-laid-down bone matrix produce the steady ionic current that returns the bone to the resting phase.

This hypothetical cycle of bone remodeling, already suggested by Marotti in 1995[28], has the advantage over the classical cycle based on undefined coupling factors, that it explains the unknown mechanism by which first osteoclast and then osteoblast cease their respective activity. The fact that osteocytes might exert an inhibitory effect throughout bone remodeling has also been suggested by Martin[29,30] on the basis of our findings on osteocyte recruitment from osteoblastic laminae[31].

In conclusion, we wish to stress that the morphometrical data here reported do not provide any information on the functional interactions occurring between osteocytes and osteoclasts. They merely show that a considerable number of osteocytes survive osteoclast resorption, thus supporting the

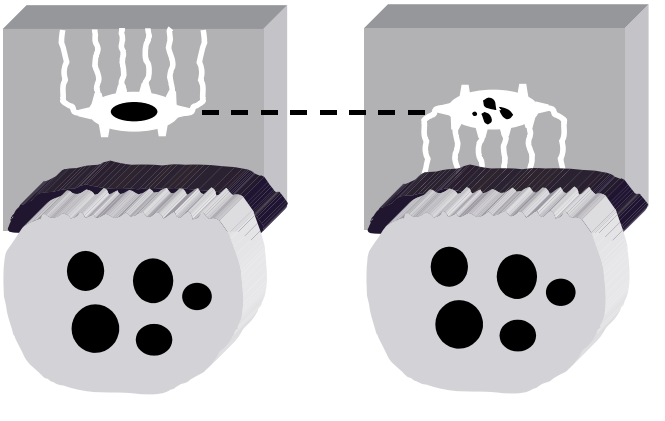

Figure 7. Schematic drawing showing two osteocytes attacked by osteoclasts on their mineral (left) and vascular (right) sides. Notwithstanding the cell bodies are at the same distance from the osteoclasts, only the osteocyte to the right shows signs of degeneration because its long vascular dendrites are affected by osteoclast disruption. See Table 1 and text for explanation.

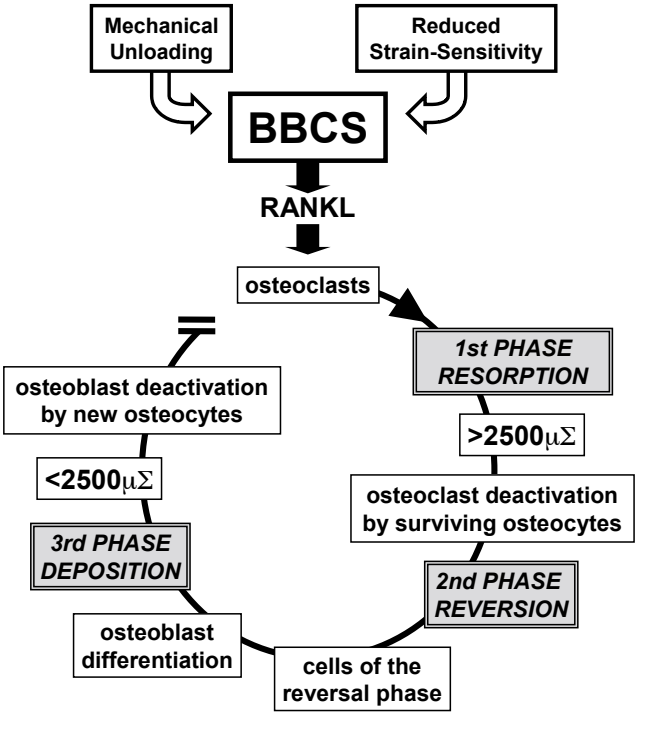

Figure 8. Postulated role of the Basic Bone Cellular System (BBCS) in bone remodeling cycle. See text for explanation.

hypothesis that osteocytes can take part in the above described mechanism involved in stopping osteoclast activity. Since neither specialized junctions nor simple cellular contacts were observed under TEM between osteocytes and osteoclasts, any modulation possibly occurring between these two cellular types can only take place by a paracrine route (*volume transmission*). This view is supported by the recent in vitro demonstration of bone resorption inhibition by an osteocyte secreted protein[32].

Acknowledgements

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