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Review

Do osteocytes contribute to bone mineral homeostasis? Osteocytic osteolysis revisited

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ABSTRACT

Osteocytes are cells buried in the bone matrix. They largely contribute to the regulation of bone remodeling in response to mechanical and microenvironmental changes. Much has been recognized in recent years regarding the role of osteocytes in bone homeostasis, nevertheless their ability to directly contribute to mineral equilibrium has been neglected. In the light of the renewed interest in their biology, we revisited the literature and discuss experimental evidence favoring the hypothesis that osteocytes are able to remove and replace the bone matrix according to the systemic needs of the body. We also reviewed reports against this theory, thus providing current views of what is known so far on the ability of osteocytes to mobilize bone mineral. This re-examination of osteocytic osteolysis might stimulate new interest and open new perspectives in osteocyte biology and in the cellular mechanisms that control bone homeostasis.

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Introduction

Osteocyte technology has been developed in recent years and has allowed significant advances in the understanding of the processes regulating bone remodeling [1–3]. These cells are now considered central to bone homeostasis [4,5] and a number of roles have been recognized, among which sensing of mechanical forces and micro-environmental conditions has been well documented [6–9]. However, several studies have provided substantial evidence that osteocytes may directly contribute to ion homeostasis by their ability to alter the perilacunar mineralized matrix. This important concept is currently neglected, therefore we have revisited the literature and discuss with a view to better understanding the biology of the osteocyte and to provide new perspectives of their role in bone homeostasis.

The osteoblast/osteocyte network

Osteocytes are spider-shaped cells buried in the mineralized bone matrix [10,11]. They provide a cellular network within the bone and are connected each other by protoplasmic extensions, at the tip of which gap junctions provide cell–cell communications [12]. Extensions form gap junctions also with the membrane of osteoblasts and lining cells located at the bone surface [12].

Many studies have suggested that osteocytes orchestrate bone remodeling, regulating osteoblast and osteoclast activities [11,13–15]. In addition, through the DMP1 [16] and FGF23[17] pathways, they form an

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endocrine organ that regulates renal phosphate reabsorption. However, early studies underlined the ability of osteocytes to directly contribute to bone calcium and phosphate mobilization [18,19], through their ability to remove [20–22] and then replace [23,24] the mineralized matrix.

Altogether osteocytes have a cellular surface estimated 400 fold greater than that of the entire Haversian and Volkmann system, and more than 100 fold greater than the trabecular bone surface [25–27]. This enormous surface has the potential to substantially contribute to bone mineral homeostasis [28]. A detectable space is present between the mineralized bone surface and the osteocyte surface, which contains non-mineralized extracellular matrix enriched in non-collagenous proteins and proteoglycans. This matrix facilitates the formation of bone fluids and the regulation of osteocyte activity through soluble factors [29]. Of note, non-collagenous proteins, located in all areas where the bone fluid is in contact with the bone mineral, have the ability to bind calcium ions [29]. Calcium phosphate, the most abundant calcium salt present in bone, has a low solubility. Binding of calcium to non collagenous proteins and phosphate solubility are reversible processes that involve an equilibrium to which osteocytes and lining cells may contribute. Interestingly, bone surfaces are completely covered by cells, and thin spaces separate them from each other, apart from the areas where gap junctions are formed [12]. Experiments performed using lanthanum injected in vivo ruled out that there is segregation between bone fluids and the rest of extracellular fluids [30]. However, regulation of ion homeostasis is likely to occur at the bone-cell surface interface, thus contributing to the equilibrium process [31,32]. Non collagenous proteins increase calcium solubility and, while not inhibiting calcium diffusion, osteocytes and lining cells may affect the time for the equilibrium between the bone fluid and the extracellular fluid to be achieved [30].

Facts that favor the bone mineral control activity of osteocytes

In 1962, Baud [33] showed by electron transmission microscopy that osteocytic lacunae have irregular borders reminiscent of osteolytic activity. Ramp and Neuman [34] favored the concept of periosteocytic osteolysis reasoning that osteoclastic bone resorption may account for only 0.1% of total calcium release, thus concluding that this is not sufficient to explain the extension of calcium regulation through bone metabolism at any given time. More information came from animal models. Periosteocytic osteolysis was observed in rats immobilized for ten days by spinal cord severing, plaster cast or ischiatic nerve dissection [35], in which destruction of lacunar wall, fragmentation of collagen fibers and loss of mineral crystals were described. These changes were abolished by thyroparathryroidectomy, suggesting the involvement of parathyroid hormone (PTH). Consistently, in thyroparathyroidectomized rats injected with moderate doses of PTH, radiolabeled calcium was rapidly released from bone in a manner that did correlate with increasing transport through the osteocyte-lining cells complex from the bone fluid compartment rather than through osteoclast bone resorption [36]. In a similar model of chronic PTH treatment in rats [37], osteocytes appeared morphologically activated, with prominent Golgi apparati, lysosome exocytosis and periosteocytic osteolysis, more pronounced at one pole of the cell. Continuous infusion of PTH in rats by a mini pump for 4 weeks, induced changes in cortical bone osteocytes consistent with periosteocytic osteolysis [38]. These cells presented with lacunae significantly larger than vehicle-treated controls, with histochemically apparent tartrate resistant acid phosphatase activity, suggestive of pericellular osteolysis caused by lysosomal enzymes. Consistent with possible osteocytic osteolysis, Lane et al. [39] showed that the osteocytic lacuna midsection area is significantly larger in mice treated with prednisone compared to placebo-treated animals and to mice subjected to ovariectomy.

In humans, a tetracycline-based histomorphometric evaluation of iliac crest biopsies of patients affected by hyperparathyroidism versus patients affected by hyperthyroidism, revealed the PTH, but not thyroid hormone(s), stimulated periosteocytic osteolysis [40]. Bernard and Meunier [41] used morphometric analysis of periosteocytic osteolysis for diagnosis of hyperparathyroidism. Cramer et al. [42] observed osteocytic activity and bone lining cell stimulation near tumor growth in osteolytic metastases from lung cancer. Bonucci [43] described the ultrastructure of osteocytes adjacent to bone metastases, noting the presence of coastal crystals along the border of the lacunae except in areas reminiscent of osteolytic activity. Many confluent osteocytic lacunae were also observed in these subjects, suggestive of a process of periosteocytic osteolysis. In bone biopsies of patients affected by renal osteodystrophy [44] or in hemodialyzed uremic subjects [45], some osteocytes were found to be located in unusually wide lacunae, showing evidence of osteolytic activity indicated by irregularity of the lacunar wall, presence of flocculent, granular and filamentous materials in the pericellular space and calcifications of mitochondria. In addition, periosteocytic osteolysis was described by Bélanger et al. [46] in patients affected by Paget's disease. More recently [47], intense periosteocytic osteolysis was described in iliac crest biopsies of monkeys subjected to 14-days spaceflight, which displayed prominent bone loss.

Periosteocytic osteolysis has been described also in many other vertebrates, both non mammalian and mammalian. In the snake *Vipera aspis*, enlargement of osteocytic lacunae, attributed to osteolytic activity, was observed in winter and, in breeding females, during the period of embryo development [48]. In these animals, no internal bone remodeling occurs during the seasonal cycle, therefore at this time there are no newly formed osteocytes that may appear larger than average. In addition, these enlarged osteocytes show a perilacunar area of decreased mineral density, denominated demineralization halo, reminiscent of mineral resorbing activity. Hibernating female brown bats were observed to have increased periosteocytic osteolysis during lactation [49], while golden hamsters and ground squirrels [50] show loss of bone during hibernation especially due to periosteocytic osteolysis [51], thus suggesting that osteocytic activity in these animals may be significant for calcium regulation in various phases of their lifespan. Cortical bone periosteocytic osteolysis was also observed in green iguanas fed an experimental low calcium/normal phosphorus diet in captivity [52]. Finally, sexual maturity in female eels was found to induce bone decalcification with hypercalcemia and hyperphosphatemia, with prominent periosteocytic osteolysis [53].

While periosteocytic osteolysis has been supported by many groups in several animal models, a few studies have attempted to assess whether or not osteocytes may also have matrix deposition and mineralization ability [54]. Jande and Belangér [55,56] proposed that osteocytes could physiologically synthesize and then remove bone matrix components. In histological sections of cortical bone from rats subjected to chronic PTH treatment, Tazawa et al. [38] observed osteocyte lacunae containing a matrix positive to hematoxylin or metachromatic for toluidine blue, which was similar to a barely mineralized, immature bone matrix. Therefore, this structure was suggested to imply regeneration of the bone matrix around the osteocyte after the lacuna was enlarged by the PTH treatment. In an immunogold study to assess the distribution of osteopontin McKee and Nanci [57] showed that this protein lines the osteocytes and their processes. Interestingly, around some osteocytes multiple bands of osteopontin were apparent, similar to reversal lines. It is possible that these layers are reminiscent of the time when the lacuna had widened and then re-filled, suggesting that it can expand and contract multiple times during the life span of an osteocyte.

Systematic studies performed to address whether or not osteocytes are able to synthesize and release bone matrix components were performed by us using laying hens [23,24], which have a tremendously high bone turnover during the egg deposition season. In a condition of dietary calcium repletion after a depletion period, compact bone osteocytic lacunae were enriched in collagen fibrils apparently just synthesized, as suggested by [^{3}H]-proline pulse and chase experiments. They were also surrounded by alcian blue- and toluidine blue-positive materials and showed a fluorescent mineralization label by tetracycline

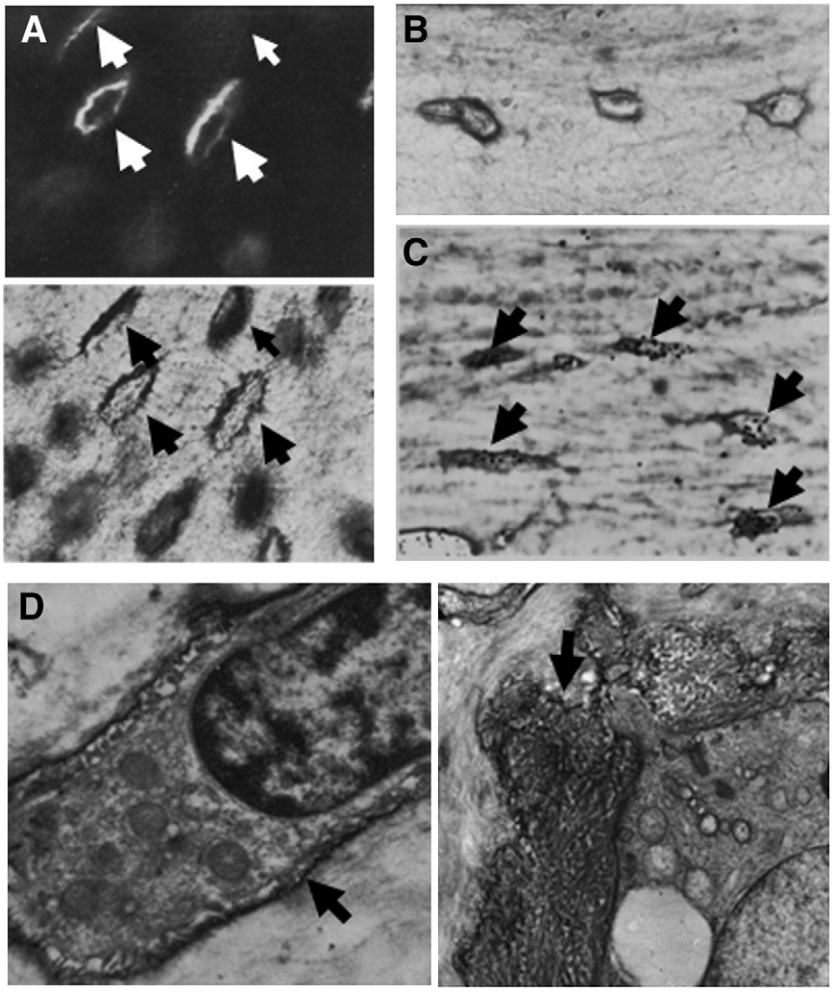

Fig. 1. Groups of White Leghorn laying hens (*Gallus domesticus*) were fed with a regular calcium diet (3% calcium) throughout the experiment, while other groups of 4 hens were subjected for 7 days to a low (0.1%) calcium diet, then returned to regular calcium feeding for 5 to 7 days, according to the experimental design. At sacrifice hens were subjected to intracardiac perfusion of phosphate buffer saline followed by 2% glutaraldehyde in phosphate buffer saline, under deep pentobarbital anesthesia, then femurs were retrieved and dissected clean, and fixation was continued. (A) Tetracycline labeling. To assess the ability to mineralize the newly formed bone matrix, animals were injected with 40 mg/kg tetracycline daily for 4 days and sacrificed 7 days after the last administration. Fluorescence (upper panel) and light (lower panel) micrographs of the same cortical bone field of a treated femoral diaphysis. Large arrows: tetracycline-positive lacunae; small arrow: tetracycline-negative lacuna. Magnification 1520×. (B) Alcian blue staining. Sections were stained with 0.1% alcian blue to detect matrix glycosaminoglycans and proteoglycans. Representative field of positive osteocytic lacunae in the cortical bone of a treated femoral diaphysis. Magnification 1440×. (C) [^{3}H]proline pulse and chase experiments. To obtain dynamic assessment of collagen synthesis, specimens from femoral diaphyses were harvested from hens at the 5th day of calcium repletion or from controls and incubated in minimum essential medium supplemented with 10% fetal calf serum and 5 μCi/ml of L-[3,4(n)-^{3}H]-proline for 3 h, followed by washing and incubation for further 3 h with excess cold proline. Semithin cross sections were subjected to autoradiography and counterstained with toluidine blue. Representative field of [^{3}H]proline-positive osteocytes in the cortical bone of a treated femoral metaphysis. Arrows: lacunae presenting with autoradiographic grains. Magnification 832×. (D) Transmission electron microscopy. Micrographs of cortical bone osteocytes from control (left panel) and treated (right panel) hens. Arrow: osmiophilic (reversal) lamina. Note low numbers of collagen fibrils and heterochromatic nucleus in control osteocyte, and high numbers of collagen fibrils and dispersed chromatin in treated osteocyte. Magnification 9300×. The pictures are reproduced from reference [24] with the permission of the Publisher.

treatment (Fig. 1). The study did not provide direct evidence of removal of bone matrix before the deposition phase. However, irregular lacunar edges outside apparently newly formed collagen fibrils were observed by transmission electron microscopy, highly suggestive of previous matrix removal, also supported by the wider distance between this edge and the osteocyte surface compared to osteocytes of control animals (Fig. 1D).

Facts against the bone mineral control activity of osteocytes

In scanning electron microscopy studies, Marotti et al. [58] proposed that the size of each osteocytic lacuna reflects the size of the osteoblast from which the osteocyte originates, with no correlation with the osteocyte activity. Boyde and Jones [59] and Boyde [60] made a case that variation of lacunar size could not occur during the life span of an osteocyte, and proposed that the morphologically apparent differences observed in many studies are artifacts merely reflecting the orientation of the cell.

In 1977, a well written report by Parfitt [61] recommended rebuttal of the osteocytic resorption and bone flow theory. According to this theory [62], bone is resorbed not from the surface by osteoclasts, but internally by osteocytes, towards which bone flows through tissue space away from bone forming surfaces. It is clear that the belief that bone can flow is incompatible with its physical properties, therefore no doubt that the flow theory is not applicable to this tissue. As far as periosteocytic osteolysis is concerned, the rebuttal was based on a number of considerations that let the Author to conclude that alternative interpretations have to be taken into account.

Technically, Parfitt argued that it has never been demonstrated that lacunar enlargement is due to cell shrinkage during specimen preparation for alpharadiography, the most popular method used in the past to demonstrate the periosteocyte osteolytic phenomenon. The fact that larger lacunae are apparent only in treated/pathological bone and not in controls was suggested to be due to an abnormal chemical and physical state of the perilacunar matrix. He also argued

that the procedure has never been calibrated and could detect reduction in density rather than complete mineral removal from the matrix. Plastic embedding, extensively used for similar purposes, was also suggested to create artifacts and lacunar enlargement in pathological bone was again attributed to the fact that the matrix around the osteocytes is more labile than normal. However, he did not comment on the reason why pathological perilacunar matrices are labile, nor was the involvement of osteocyte activity in this context taken into account. He also stated that increased size of osteocytic lacunae has been often misinterpreted, especially considering that they are larger at the osteon periphery than at its center, reflecting the original size of osteoblasts from which they arise, thus supporting the report by Marotti et al. [58].

Further evidence for rebuttal relates with the fact that, according to complex histomorphometric calculations, total osteocytic resorption in the tibia of growing rats was estimated to be one tenth of the resorbing capacity of osteoclasts [63], insufficient to guarantee efficient ion homeostasis. This contradicts the report by Ramp and Neuman [34] who affirmed that osteoclastic bone resorption may account for only 0.1% of total calcium release, thus concluding that periosteocytic osteolysis is necessary for efficient ion homeostasis.

Furthermore, Parfitt acknowledged that it is difficult to explain how the products of osteolysis are to be removed [64] as the fluid movement through the lacuno-canalicular system may be insufficient as well [65,66], or could even be hindered in extra Haversian bone, in which canaliculi do not generally communicate with those of the osteon [64]. However this did not take into account that extra Haversian osteocytes were not observed in the phase of osteolytic activity at any given time. In addition, it is not clear how extra Haversian osteocytes can keep alive if they do not communicate with the Haversian systems, given that lanthanum experiments ruled out segregation between bone fluids at the matrix–osteocyte interface and the rest of extracellular fluid [29,30].

Perspectives

We have herein detailed the studies *pro* and *con* the ability of osteocytes to remove and replace significant amount of bone matrix to become relevant for ion homeostasis. Numerous reports favored while only a few argued against this hypothesis, focusing their rebuttal on data interpretation rather than *ad hoc* experimental evidence.

Our conclusion is more in favor than against osteocytic mineral control ability, and we agree with Wassermann and Yaeger [67] who suggested that, in this context, physiologic activity of osteocytes could occur sub-microscopically, perhaps involving the coastal crystals observed in the perilacunar matrix, with no morphological changes in lacunar size and shape. It is only in pathological conditions (hyperparathyroidism, renal osteodystrophy, hemodialyzed uremic patients, bone metastases) that the phenomenon become morphologically recognizable, a situation that can be reproduced in animal models in

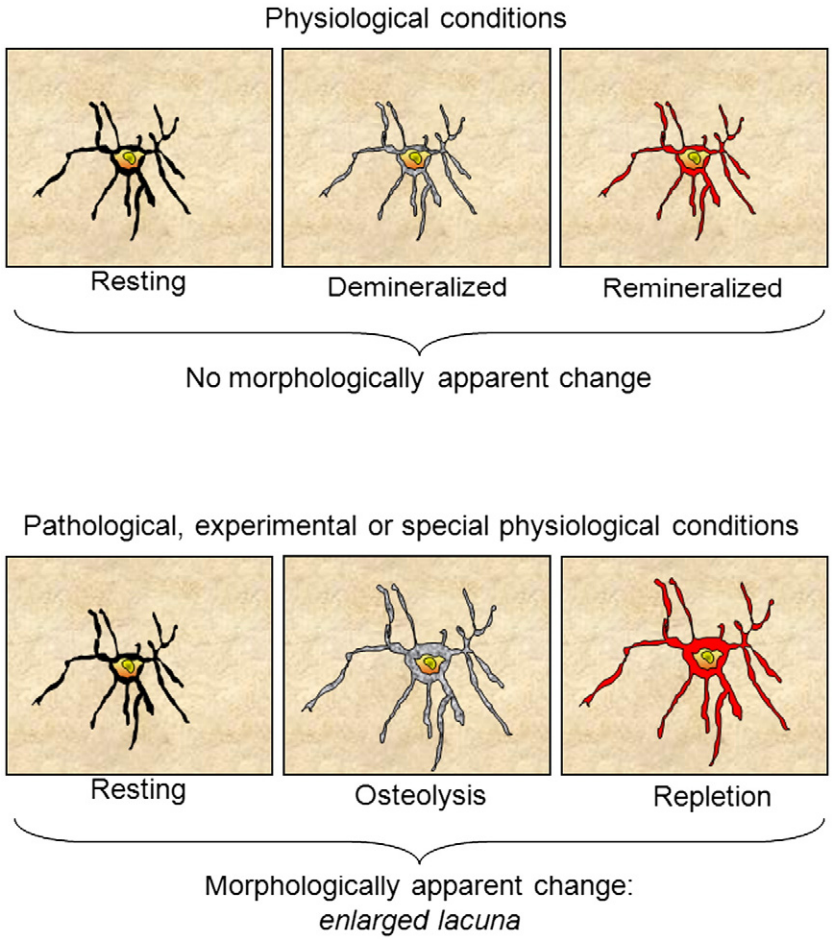

Fig. 2. Cartoon showing our perspective on how an osteocyte could perform physiological (upper panels) and pathological or experimental (lower panels) bone mineral control activity. In the former, periosteocytic bone could be demineralized (grey color) or remineralized (red color) various times to rapidly contribute to the calcium and phosphorous balance, with no morphologically apparent changes. In this circumstance, the structure of the bone would remain unaltered. In the latter, when the demand for calcium and phosphorus is increased by pathological or experimental conditions, or due to special situations, including hibernation, pregnancy and lactation, lacunar and, perhaps, canalicular spaces are enlarged during the osteolytic phase. The same osteocyte could replace and mineralize the newly formed bone matrix at the end of the depletion period. This process may leave minimal morphologically evident changes, which however are unlikely to alter the mechanical properties of the bone.

particular physiological circumstances (i.e. in animals with seasonal activities, or during pregnancy or lactation) or by experimental manipulations (i.e. PTH or glucocorticoid treatment, low calcium intake) (Fig. 2).

We are fully aware that most experimental evidence originated from early studies principally based on morphological *in vivo* observations. However, it is amazing how the osteocyte field was evolving at that time and how well controlled were the studies considered in this report. Interpretation of the results could be an issue, as there were no cellular and molecular techniques readily available that could definitely provide clear responses to this important aspect of bone homeostasis. However, we believe that current osteocyte technology and the ability to genetically manipulate animal models should allow experiments that provide definitive insights into the ability of osteocytes to mobilize bone mineral. This may have an important impact for the understanding of the pathophysiology of bone diseases and for the identification of new therapeutic tools for both pharmacological and regenerative medicine.

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