Transmission electron microscopy of bone tissue

A review

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Remodeling permits the bone to preserve its static and dynamic functions. If this process is disturbed the bone becomes more susceptible to fracture. Remodeling also allows the bone to play a major role as a bank of mineral substances, mainly calcium and phosphorus.

The remodeling activities, a very precise sequence of events, concerns compact bone tissue of cortices, as well as cancellous bone tissue. After activation, leading to the birth of osteoclasts from hemopoietic stem cells, there is an osteoclastic resorption forming Howship lacunae. Then, and following a reversal phase, osteoblasts come from osteoprogenitor cells and rebuild bone tissue during the formation phase. There is a coupling, in space and time, between resorption and formation activities (Frost 1973).

The purpose of this paper is to review the transmission electron microscopy aspects of bone tissue in order to evaluate (a) the composition of calcified and not yet calcified matrix, (b) the structure and role of osteoclasts and osteoblasts, (c) the aspects of bone cells not directly involved in bone remodeling, such as osteocytes and lining cells.

Materials and methods

Knowledge about the ultrastructural features of bone tissue stems from examinations of undecalcified bone tissue samples, mainly the iliac crest, which is the most commonly used sampling site in humans. The samples should be cut into 1 to 2 mm^3 small pieces to facilitate their immediate fixation and subsequent embedding.

A lot of procedures have been used to prepare bone tissue for transmission electron microscopy. Several types of fixation, dehydration, embedding, and staining procedures have been described (Landis et al. 1977a, b, 1980, Boivin et al. 1983, 1987b, 1989, Boyde 1984, Dickson 1984a, b). A re-embedding method has also been developed (Boivin and Baud 1984) permitting the ultrastructural observation of limited zones of sections previously studied by means of microradiography and light microscopy. It is evident that these latter fixation methods do not permit a fine analysis of the cells; but they are sufficient, however, for the purpose of obtaining an image of certain aspects of the mineral substance (e.g., shape, size, or distribution of the crystals), as well as their relationships with the organic matrix (mostly collagen fibrils). This technique is useful because the area studied is rigorously selected and oriented allowing certain details to be studied thoroughly, for example, the aspects of the periosteocytic lacunae in experimental or pathologic conditions (Baud and Boivin 1978a, b).

Finally, the method currently used for our human bone samples is briefly described. Cancellous or cortical bone samples taken at biopsy are immediately fixed for 2 hours in glutaraldehyde (2.8 percent in sodium-cacodylate buffer 0.1 M), washed overnight in a saccharose solution (0.2 M in the same buffer), and then postfixed 1 hour in osmium tetroxide (1 percent in the same buffer). After dehydration in alcohol and propylene oxide, the samples are embedded in epon B. Semithin sections, cut with a glass knife and stained with toluidine blue, are used to localize the zones of interest for electron-microscopic studies. Ultrathin sections are then cut on an ultramicrotome equipped with a diamond knife. The sections are now ready to be stained with uranyl acetate and lead citrate, and examined on a transmission electron microscope.

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Figure 1. Organic matrix of bone is mainly composed of collagen type I fibrils showing, in longitudinal section, a regular cross-banding. × 30,000.

Figure 1.

Figure 2. Osteoid tissue (O), the uncalcified organic matrix of bone (here, transversally cut collagen fibrils), is secreted by active osteoblasts (OB). × 9,000.

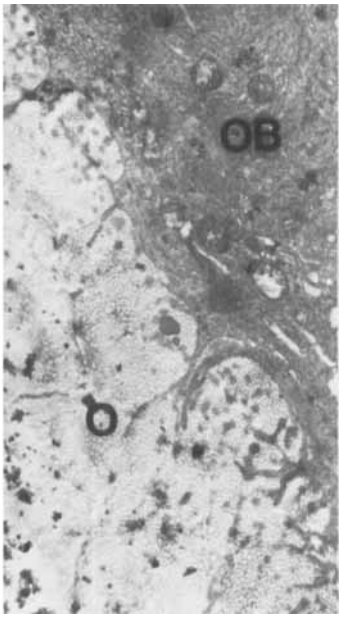

Figure 2.

The organic matrix of bone tissue

This matrix, rigid in consistency, is essentially composed of collagen fibrils and an interfibrillar ground substance.

The most abundant protein of the organic matrix of bone is type I collagen (90 percent), the other proteins representing only 10 percent (Bonucci 1984, Veis and Sabsay 1987, Marks and Popoff 1988). Most of the organic matrix of bone is produced by the osteoblasts. Collagen type I fibrils have a diameter ranging between 50 and 70 nm and a characteristic axial periodicity of about 68 nm (Figure 1). The collagen fibrils may be observed in osteoid tissue, i.e., the more or less thick layer of not yet mineralized organic matrix that separates calcified bone tissue from osteoblasts or bone lining cells (Figure 2). In mineralized bone tissue, their structure is normally masked by the mineral substance and can therefore only be observed after decalcification. In lamellar bone tissue the collagen fibrils are closely packed and often oriented parallel to each other. This orientation appears to be more regular in lamellae of compact bone than in cancellous bone. An ultrastructural study of lamellar organization in human bone has recently been published (Reid 1986). In woven bone tissue, such as callus formation, collagen fibrils show no orientation, the fibrils are haphazardly intermingled, and the amount of ground substance encountering the fibrils appears more abundant than in lamellar bone tissue. In osteoid tissue, the degree of aggregation and arrangement of collagen fibrils increases in the vicinity of the calcifying matrix, i.e., close to the calcification front (Fornasier 1977, Bonucci 1984). In compact bone of human osteons, collagen fibrils have been shown to be arranged according to a twisted plywood architecture (Giraud-Guille 1988). Finally, from a structural point of view, collagen fibrils in bone resemble those observed in other connective tissues, whether calcified or not. Extremely small amounts of other types of collagen (II, III, V, X) have been detected in bone matrix, but their presence is generally explained as a contamination or is due to a pathologic condition in the bone matrix. For example, types I and III collagens have been found in bone matrix from patients suffering from osteogenesis imperfecta (Muller et al. 1977, Pope et al. 1980, Boivin et al. 1984).

The extrafibrillar ground substance of bone matrix is composed of numerous constituents, mainly noncollagenous proteins (Fisher and Termine 1985, Fisher et al. 1987, Tracy et al. 1987, Triffitt 1987, Marks and Popoff 1988). Some of these proteins have been extensively studied as potential markers of bone turnover. Our knowledge about this ground substance is mainly based on data from biochemical investigations. Some of our present knowledge concerning these proteins also stems from electron microscopy findings in conjunction with immunocytochemistry.

Osteonectin, a recently discovered non-collagenous protein in bone, is a phosphoprotein synthesized by osteoblasts, but it is not a specific bone protein (Termine 1983, Jundt et al. 1987, Bianco et al. 1988, Tracy et al. 1988). Osteocalcin, or bone Gla protein, is vitamin K dependently synthesized by osteoblasts. This protein contains three residues of the calcium-binding amino acid gamma carboxyglutamic acid (Price 1985, Hauschka 1986, Lian and Gundberg 1988). Osteocalcin has recently been localized in osteoblasts and bone matrix by using ultrastructural immunocytochemical methods (Groot et al. 1986, Boivin et al. 1987a, Bronckers et al. 1987, Carmada et al. 1987, Mark et al. 1987). Osteocalcin is, to a small part, released into the blood where it can be measured with radioimmunoassay as a marker of bone turnover (Price et al. 1980, Delmas 1987, Epstein 1988). Another Gla-containing noncollagenous protein (matrix Gla protein) has recently been identified and contains five gamma-carboxyglutamic acid residues. However, in numerous tissues, this protein appears more widely distributed than osteocalcin (Price et al. 1983). Moreover, two small proteoglycans concentrated near the calcification front have also been described (Fisher 1985, Nefussi et al. 1989). Finally, bone matrix also contains bone sialoproteins I (= osteopontin ; Mark et al. 1987) and II, various other glycoproteins and phosphoproteins, and a large variety of growth factors (Marks and Popoff 1988).

Figure 3. The needle-like crystals composing the bone mineral substance (B) are arranged between and/or in collagen fibrils. × 15,000.

The mineral substance of bone tissue

Bone mineral substance is a microcrystalline analogue of the mineral hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$, with a calcium deficiency of about 5 to 10 percent. This deficiency is balanced by a combination of missing hydroxyl ions and hydrogen bonds between oxygens of orthophosphate (Posner 1985, 1987). Stoichiometric hydroxyapatite is neither calcium- nor hydroxyl-deficient, and does not contain hydrogen bonds. The bone mineral substance also contains 3 to 4 percent carbonate ions instead of phosphate ions. The presence of carbonate induces a misalignment in the bone mineral crystal, provoking an increase in its chemical reactivity. Bone crystals show a highly specific surface and have reactive surfaces (Posner 1987), allowing for numerous ionic substitutions (fluoride in particular).

The ultrastructure of bone mineral substance is not absolutely constant; it partly depends on texture, structure, and degree of mineralization of the bone tissue (Bonucci 1984). Extensive studies by x-ray diffraction and transmission electron microscopy have been performed on crystal shape, size, and orientation, and the results of these studies show a wide variation. As a summary, bone mineral substance appears as needlelike and platelike crystals 1.5 to 5 nm thick, 5 to 10 nm wide, and 50 to 100 nm long (Bonucci 1984, Glimcher 1984). Recently, Arsenault and Grynpas (1988) have reported data on cortical bone of the rat, using x-ray diffraction and electron microscopy (bright-field and selected-area dark-field imaging). These authors found needlelike crystals 12 to 17 nm in length and 5 nm in width; they also reported that the platelike structures would in fact be aggregates of small needlelike crystals.

Bone mineral crystals (Figure 3) may be observed either outside or inside the collagen fibrils. In the latter case, crystals are placed in the hole zones of fibrils thus reinforcing their regular cross-banding (Bonucci 1984, Glimcher 1984, Lees and Prostak 1988). After postembedding decalcification, Bonucci (1984, 1987) has described organic components called crystal ghosts. They have the same shape and size as crystals and would be lipoglycoproteins in nature.

Figure 4. The osteoclast, a bone resorbing cell, is a large, multinucleated cell. × 2,300.

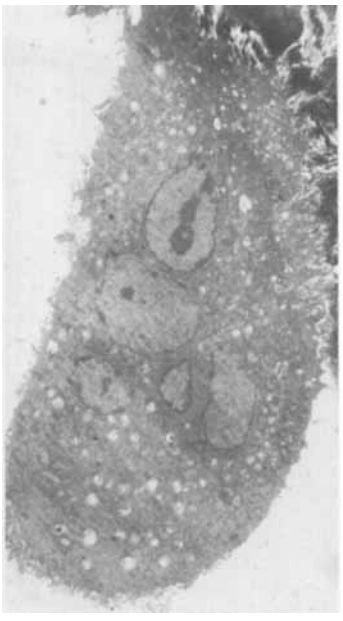

Figure 4.

Figure 5. The ruffled border (R), the active site of resorption, is in close contact with bone tissue (B). In the vicinity of the ruffled border, collagen fibrils appear more or less decalcified (arrow). × 15,000.

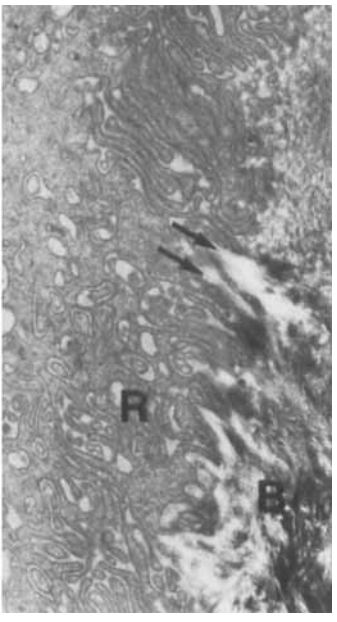

Figure 5.

The osteoclasts

The ultrastructural aspects of osteoclasts have been extensively described (Holtrop and King 1977, Lucht 1980, Bonucci 1981, Marks 1983, Chambers 1985, Baron et al. 1986, Nijweide et al. 1986, Mundy and Roodman 1987, Marks and Popoff 1988, Vaes 1988). Osteoclasts are large, multinucleated cells capable of penetrating the bone tissue (Figure 4). When they are active and resorb bone tissue, it is possible to identify two specializations of their plasma membrane (a clear zone and a ruffled border) located in the vicinity of or in close contact with the bone tissue. The ruffled border (Figure 5), the active site of resorption, consists of deep invaginations of the cell membrane in the vicinity of bone tissue to be resorbed (Domon and Wakita 1986). The clear zone is almost totally free of cell organelles and encircles the ruffled border. The nuclei are distributed in the cytoplasm at a certain distance from the resorbing area, and are associated with a Golgi apparatus. Concerning the cytoplasm, one of the most striking features is that it contains numerous mitochondria and prominent vacuoles often close to the ruffled border. These vacuoles are lysosomes and allow the cell to be a true enzyme factory functioning as a recycling center (Marks and Popoff 1988). Acid hydrolases packaged in the perinuclear Golgi apparatus are moved to the ruffled border region then ejected outside the cell into the extracellular space confined between the bone tissue and the tight seal laterally provided by the clear zone between bone and cell. Thus, in this space the bone tissue is degraded in an acid environment. The degradation products are taken up in digestive vacuoles and lysosomes, further degraded, and released into neighboring vascular spaces. Thus, bone resorption involves exocytosis and endocytosis at the ruffled border. The osteoclast progenitors are mononuclear cells that arise in hemopoietic tissues and travel to the bone microenvironment via the circulation, where they differentiate and fuse to form osteoclasts. The monocyte-macrophage lineage constitutes a good candidate for the origin of osteoclasts (Marks and Popoff 1988). When the resorbing activity is ended, the specializations of the cell membrane progressively disappear and the osteoclast separates from the bone tissue.

Some bone pathologies are mainly characterized by different types of osteoclast incompetences in which electron microscope observations have been of particular value in elucidating them. In particular, such is the case in osteopetrosis where several alterations of osteoclast function have been described (Marks 1984), and in Paget's disease of bone where a viral origin of this disease seems likely (Baslé et al. 1987, Rebel et al. 1987).

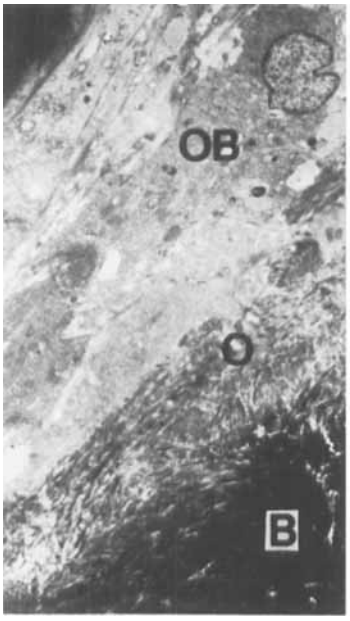

Figure 6.

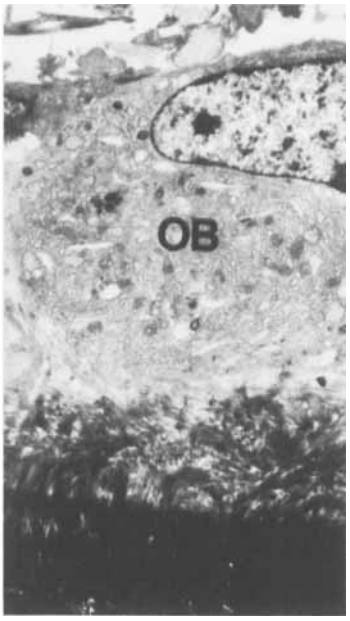

Figure 7.

Figure 6. Active osteoblasts (OB) are separated from calcified bone tissue (B) by a layer of uncalcified organic matrix, i.e., osteoid tissue (O). × 3,000.

Figure 7. An active osteoblast (OB) containing a prominent rough endoplasmic reticulum, a cytoplasmic organelle involved in organic matrix synthesis. × 4,500.

The osteoblasts

These are small, mononucleated cells derived from local, nonmigratory, undifferentiated mesenchymatous cells of the bone marrow (Friedenstein 1976, Owen 1985, Marks and Popoff 1988). The osteoblasts (Figure 6) are involved in the function of bone formation (Holtrop 1975, Marks and Popoff 1988). They synthesize bone matrix constituents (Figures 2 and 7), i.e., osteoid tissue, and control the calcification of the organic matrix. The kinetic aspects of bone matrix synthesis and of its calcification have been particularly well demonstrated using autoradiography with labeled amino acids and calcium (Frank 1979).

While all osteoblasts are osteogenic in nature, their shape, size, and arrangement suggest subpopulations exhibiting different levels of activity (Zallone 1977, Ries et al. 1985). Numerous osteoblasts are very active, have a cubic shape (plump osteoblasts), and are arranged perpendicularly to the bone surface. Their nucleus is disposed in the part opposite to the bone surface. Their cytoplasm shows all the features of cells synthesizing proteins for export, i.e., an abundant and often dilated rough endoplasmic reticulum, and a very well developed Golgi apparatus (Figure 7). Other osteoblasts are more or less flattened along the bone surfaces (flat osteoblasts), and they appear to be less active than the plump ones, as proven by the lesser development and extension of their cytoplasmic organites. The ultrastructural aspects of these two types of osteoblasts have recently been observed in a case of skeletal fluorosis (Boivin et al. 1989). Mitochondria are often numerous in the cytoplasm of osteoblasts. The presence of mineral deposits in mitochondria as intramitochondrial granules has been extensively reported in various tissues, including calcifying ones (Boskey 1981). However, it is still unclear whether the mitochondrial granules observed in osteoblasts (Manston and Katchburian 1984) are involved in the calcification mechanisms.

At the surface of bone formative zones, the osteoblasts are not isolated but are arranged side by side, thus constituting an alignment of osteoformative elements along the bone surface. These osteoblasts are electrically coupled cells, having conductance pathways and communication between them (Jeansonne et al. 1979). Different types of cell junctions have been described between osteoblasts, such as tight and gap junctions (Weinger and Holtrop 1974, Stanka 1975, Marquart 1977, Doty 1981). These cell membrane specializations would seem to play a major function in the control and coordination of osteoblast activity at the level of a formation functional unit responding coherently and homogeneously to various stimuli.

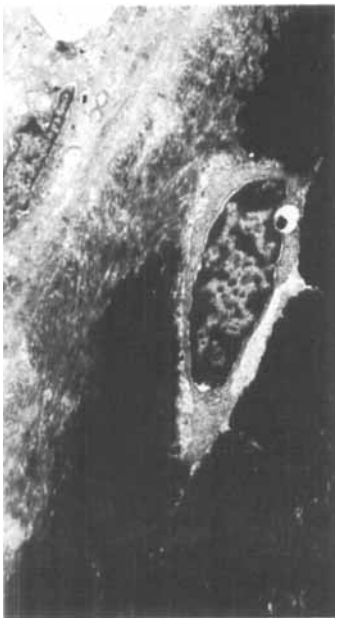

Figure 8. Certain osteoblasts are progressively surrounded by organic matrix then by calcified tissue. Finally, when totally surrounded by calcified bone tissue, these cells are called osteocytes and are located in periosteocytic lacunae. × 8,500.

The osteocytes

All the osteoblasts secrete a bone matrix along calcified bone tissue during their active phase. However, some osteoblasts also secrete organic matrix all around them; thus, they are progressively surrounded by a calcified matrix (Figure 8). At this stage the bone cell is called osteocyte and is located in a periosteocytic lacuna. Sometimes, this lacuna is partly or totally surrounded by not yet calcified bone matrix and the corresponding cell is named osteoid-osteocyte (Palumbo 1986). Osteocytes are connected to adjacent osteocytes and to osteoblasts at the surface of bone tissue by numerous cytoplasmic projections located in canaliculi going through the calcified bone tissue. These cell processes are rich in microfilaments and connected by cell junctions, often tight and sometimes gap junctions (Furseth 1973, Weinger and Holtrop 1974, Holtrop 1975, Doty 1981). The periosteocytic lacunae are almost completely occupied by single osteocytes. These lacunae have the shape of flattened triaxial ellipsoids; in human iliac crest the mean values for the three principal axes are 17.4 ± 0.6 μm (± SEM), 9.1 ± 0.3 μm and 4.4 ± 0.2 μm (Baud and Boivin 1980). The periosteocytic lacunae have a regular orientation depending on the texture and structure of the bone (Marotti 1979, Baud and Boivin 1980, Cane et al. 1982). Osteocytes appear more numerous and larger in woven bone than in lamellar bone.

The osteocytes are derived from osteoblasts, but generally their nucleus appears prominent and is situated in the center of the cells. The cytoplasm appears thus less abundant than in osteoblasts, and it is mainly occupied by mitochondria, vacuoles, some profiles of rough endoplasmic reticulum, rare Golgi apparatus, glycogen. As for osteoblasts, some mitochondria contain mineral granules. The fine ultrastructural aspects and the formation of osteocytes have been mainly described by Baud (1962), Jande (1971), Tonna (1973), Luk et al. (1974), Schulz et al. (1974), Holtrop (1975).

The shape and the ultrastructure of osteocytes are not constant, but vary with their activity. Indeed, an osteocytic miniremodeling activity has been described with resorption (periosteocytic osteolysis) and formation (periosteocytic osteoplasis) phases. The changes in osteocytic activity involve modifications at the level of the perilacunar walls (enlargement or decrease in the size of the periosteocytic lacunae, respectively). The different aspects of this miniremodeling activity have been described by Baud (1968), Bélanger (1969), Tonna (1972), Jande and Bélanger (1973), Baud and Boivin (1980), Zallone et al. (1983). In summary, according to its metabolic activity, osteocytes can probably resorb bone tissue all around their lacunae, and they are also capable of forming a calcified organic matrix around their lacunae.

As regards osteoclasts and osteoblasts, osteocytes respond to the action of systemic or local factors by modifications of their activity. These modifications can often be seen in the changes that they provoke at the level of the perilacunar walls. Among the more frequent modifications of the perilacunar wall structure, there are enlargement and decrease of lacunar size, but also the formation of perilacunar halos (Baud and Boivin 1978a, b, 1980). Concerning the halos, it is important to distinguish between those created all around the lacunae following decalcification of the bone matrix (the structure of organic matrix is unchanged) and those eccentrically disposed around the lacunae and due to impaired formation (hypomineralization and defect of organic matrix). Finally, certain stimuli, like ischemia, can cause the death of osteocytes in their periosteocytic lacunae

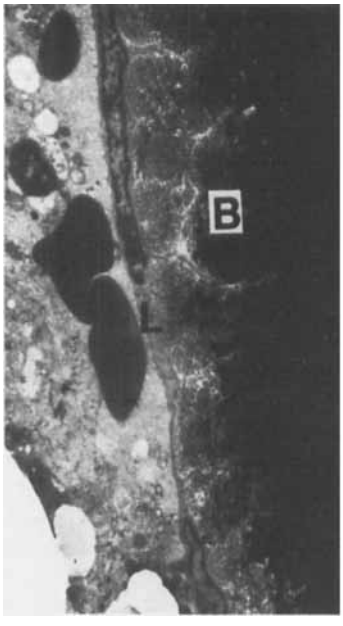

Figure 9.

Figure 9. Resting bone surfaces (B) are covered with thin and elongated lining cells (L). × 4,500.

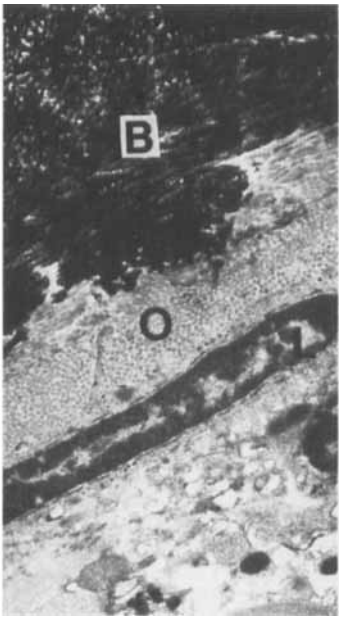

Figure 10.

Figure 10. Bone lining cell (L) shows a prominent elongated nucleus, and is always separated from calcified bone tissue (B) by a layer of osteoid tissue (O). × 15,000.

(James and Steijn-Myagkaya 1986). It is possible to distinguish between dead and living osteocytes, and thus determine, for instance, regions of bone infarction.

The bone lining cells

Under normal physiologic conditions, resting bone surfaces are covered with very elongated and thin lining cells (Figure 9). These cells are extremely flattened along the bone surface and sometimes can only be observed under electron microscopy. Their nucleus is prominent and occupies a large part of the cell (Figure 10), while their cytoplasm is less abundant and poor in mitochondria, vacuoles, and ribosomes (Vander Wiel et al. 1978, Miller et al. 1980, Miller and Jee 1987). The bone lining cells are to be distinguished from the so-called flat osteoblasts that are true but less active osteoblasts (Boivin et al. 1989).

The bone lining cells separate bone tissue from bone marrow but these cells are always separated from bone by a narrow layer of osteoid tissue (Figures 9 and 10). The roles of the bone lining cells are not well established. They could control the regulation of mineral homeostasis (Norimatsu et al. 1979). Indeed, the almost constant association of these cells with capillaries near bone surfaces suggests that they may have a role in partitioning bone fluids from extracellular fluids (Miller and Jee 1980). Bone lining cells, as a part of the cells of the osteoblast lineage, may also be involved in the initiation and activation processes of bone remodeling, and thus promote the recruitment of osteoclasts and/or remove the uncalcified layer of organic matrix which protects bone tissue (Miller and Jee 1987).

Concluding remarks

Electron-microscopic examination of bone tissue has, from a clinical point of view, no great importance as yet. Bone pathology is never diagnosed using electron microscopy alone. In the future, however, electron microscopy alone or in association with complementary techniques, such as autoradiography, immuno cytochemistry, microanalysis, and cell culture, will be valuable and needful to precisely describe bone pathologies. Some examples concerning osteogenesis imperfecta, osteopetrosis, Paget's disease of bone have already been mentioned in the present paper. For the evaluation of bone tissue reactions to different implant materials, electron microscopy has only recently come into use (Linder et al. 1989). Synchronous with the development of im-

paired technical equipment for the sectioning of hard tissue and metals, this field of orthopedic clinical research should become of increasing interest.

Numerous systemic or local factors (hormones, vitamins, growth factors, ions, etc.) are involved in the control or modification of the osteoblast and osteoclast functions and activities (Rodan and Martin 1981, Sakamoto and Sakamoto 1982, Canalis 1983, Raisz and Kream 1983, Chambers 1987, Marks and Popoff 1988). Electron microscopy will be very useful for obtaining observational precision as regards the tissular and cellular changes involved by these factors. For example, mechanisms of bone cellular effects of fluoride, mainly used as a treatment of type I osteoporosis, have to be elucidated (Boivin et al. 1989, Boivin and Meunier in press).

Finally, in view of the increasing age-adjusted incidence of fragility fractures (Obrant et al. 1989), it is necessary not only to establish conditions with gross pathology, but also minute changes in the mineral composition and structure, evaluated by microanalysis (Obrant and Odselius 1984, 1985) and x-ray diffraction (Arsenault and Grynpas 1988), respectively, will become of increasing interest.

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