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An Electron Microscopic Investigation of Human Familial Bone Dysplasia

Inhibition of Osteocytic Osteolysis and Induction of Osteocytic Formation of Elastic Fibers Following Calcitonin Treatment

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Familial bone dysplasia with hyperphosphatasemia is characterized by excessive bone resorption early in life with resulting severe skeletal deformity. The disease can be ameliorated by treatment with human calcitonin. We have studied the ultrastructure of bone from diseased patients before treatment and at intervals during 1 year of treatment with calcitonin. Pretreatment osteoblasts, osteoclasts, and osteocytes exhibited mitochondria which contained vast amounts of dense microcrystal deposits. Osteocytes were also distinguished by minimal organellar development. Osteoclasts were rare. Calcitonin treatment included a progressive development of a more normal bone structure. Intramitochondrial crystal deposits were absent in mitochondria of osteocytes and osteoclasts but were still present in mitochondria of osteoblasts. Surprisingly, the developing bony matrix during calcitonin treatment exhibited large numbers of elastic fibers. These appeared to develop normally in alignment with the surface membrane of osteocytes. Calcitonin treatment caused a proliferation of osteocyte organellar development. It is concluded that familial bone dysplasia is primarily a disease of osteocytes and that osteocytic activity is influenced by calcitonin. (Am J Pathol 94:1–18, 1979)

TWO RESORPTIVE PROCESSES have been found to play a role in the modeling of bone: osteocytic osteolysis and osteoclasia. The inter-

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action and the particular function of each remain enigmatic and difficult to sort experimentally. Familial bone dysplasia with hyperphosphatasemia is a rare bone disease which involves excessive bone resorption early in life, resulting in severe bone deformity.[1-3] Thus, human familial bone dysplasia provides an experiment of nature that may help in understanding these processes. We have found that the disease can be ameliorated by treatment with human calcitonin. In the patients we have studied, calcitonin treatment reduced bone pain and tenderness while increasing the patients' mobility. Serum alkaline phosphatase and urinary hydroxyproline levels were greatly reduced.[4] We have also reported the progressive radiographic and histologic changes which take place during the course of 1 year of calcitonin treatment. Prior to treatment, bone lacked a distinct separation between the cortex and the medullary cavities when examined radiologically, but following treatment a discrete compact cortex clearly formed.[5] Histologic examination of the appearance of bone in the disease state revealed that the excessive resorption appeared to be due primarily to osteocytic osteolysis. Osteoclasts were extremely few and were normal in appearance, indicating that they probably play a minor, if any, role in the disease process. Calcitonin treatment markedly inhibited osteocytic osteolysis and induced a change in bone structure from woven bone to lamellar bone.[5] To further define the action of calcitonin on the diseased bone, we have examined in detail the ultrastructure of the diseased bone before and during the course of calcitonin therapy in two siblings with the disease. The results of the study are reported here.

Materials and Methods

Two siblings with the disease were studied. The siblings, a 4-year-old male (MA-31) and a 7-year-old female (BL-30), were hospitalized in the metabolic unit of the Institute of Nutrition of Central America and Panama (INCAP). They were maintained on a low-proline diet calorically adequate for their height and weight. The 2 patients were treated with synthetic human calcitonin (Ciba-Geigy), 0.5 mg bid.

Open iliac crest biopsies were carried out on the siblings just prior to the onset of calcitonin therapy and at 4.5 months, 6 months, and 1 year of hormone treatment. The biopsy specimens were carefully taken to include the trabeculae immediately beneath the surface of the bone (primary trabeculae) and the trabeculae deeper (secondary trabeculae). This allowed comparison of these same areas before treatment and after treatment.

The biopsy samples were cut into small pieces which were immersed in cold 6.25% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 48 to 96 hours. The tissues were then washed in ice-cold 0.25% sucrose in 0.067 M cacodylate buffer and were postfixed in ice-cold 1.0% osmium tetroxide in 0.067 M cacodylate buffer, pH 7.3, for several hours. All tissues were dehydrated and embedded in epon. Thin sections were doubly stained with uranyl acetate and lead citrate and were examined with a Philips EM-300 electron microscope.

The histologic appearance of the lesion is illustrated in Figures 1A and B. Briefly, the trabeculae of the affected children were characterized by an intense osteocytic osteolysis (osteocytes and their lacunae were enlarged), the absence of cartilaginous core, and the

presence of numerous cementing lines and of thick osteoid seams. There was also a moderate fibroplasia between the trabeculae. When viewed with polarized light, the histologic sections revealed poorly organized collagen fibrils. At the ultrastructural level, in general, pretreatment bone was very poorly mineralized, with only scattered areas of calcification. Prior to treatment with calcitonin, collagen fibrils were loosely arranged and highly disoriented. Calcitonin treatment resulted in the appearance of normal, well-organized extracellular collagen fibrils, but the extracellular matrix still appeared, to a great extent, hypomineralized.

Results

Osteoblasts and osteocytes were routinely found in pretreatment and posttreatment bone specimens, but osteoclasts were extremely rare. Only several osteoclasts were observed in pretreatment and posttreatment bone samples. A description of the major ultrastructural features of the different bone cell types prior to and during calcitonin treatment is described below. Also, the unique appearance of elastic fibers in the extracellular matrix following calcitonin therapy is presented.

Osteoblasts

Precalcitonin Treatment

Cuboidal to elongated osteoblasts were commonly found in thin sections. Ultrastructurally, the cytoplasm of osteoblasts before calcitonin treatment was characterized by a well-developed rough-surfaced endoplasmic reticulum. In some cells, the granular endoplasmic reticulum consisted of many dilated to slightly dilated profiles scattered throughout the cytoplasmic matrix. In other cells, however, it consisted of stacks of parallel flattened to slightly dilated membranes. A well-developed Golgi complex, many medium to large vacuoles, and occasional dense lysosomal bodies were also found in the cytoplasmic matrix. A normal-appearing nucleus was usually found in the center of the cell profile (Figure 2).

A most distinctive feature of the osteoblast was that mitochondria often contained various numbers of electron-dense microcrystal bodies (Figures 2 and 3). Otherwise, the mitochondria appeared normal. Mitochondria were spherical or cigar-shaped, approximately 0.5 to 1.0 μ in size, and bounded by a double membrane (Figure 3). The intramitochondrial needle-shaped microcrystal deposits were sometimes located near or over cristae, but usually they were found in the mitochondrial matrix (Figures 2 and 3). Microcrystal deposits were rarely found over the outer mitochondrial limiting membrane. It is interesting that mitochondria from non-bone cells in the specimen, such as marrow cells, never exhibited mitochondrial microcrystal deposits.

Postcalcitonin Treatment

Osteoblasts following calcitonin therapy did not differ ultrastructurally from those of the pretreatment tissue. The cells continued to exhibit a cytoplasm which contained an extensive granular endoplasmic reticulum, a well-developed Golgi complex, many prominent vacuoles, and abundant mitochondria. Mitochondria still contained prominent dense microcrystal deposits (Figure 4).

Osteoclasts

Pretreatment osteoclasts were characterized by a cytoplasmic matrix filled with mitochondria, the majority of which contained dense intramitochondrial microcrystal deposits (Figure 5). Usually, one or two normal-appearing nuclei were also found in the central region of the cell profile (Figure 5). Calcitonin treatment resulted in the disappearance of the intramitochondrial microcrystalline deposits (Figure 6). Otherwise, no other major ultrastructural change was noted following hormonal treatment.

Osteocytes

Precalcitonin Treatment

Osteocytes were typically found in wide pericellular spaces which contained a flocculent material but very few collagen fibrils (Figure 7). Ultrastructurally, osteocytes found in such spaces contained a large, slightly irregular nucleus. A rim of condensed chromatin was found around the periphery; the central portion of the nucleus contained scattered clumps of chromatin. Cytoplasmic organellar development was minimal. The Golgi apparatus, when encountered, was relatively small. Most osteocytes showed only a few cisternae of the granular endoplasmic reticulum (Figure 7). Lysosome-like dense bodies and vacuoles were found in the cytoplasm of most cells. The number of mitochondria in the cytoplasm varied from cell to cell. Most cells, however, contained only a few. The majority of the mitochondria were almost completely filled with electron-dense microcrystal deposits. Such mitochondria appeared as solid, dense bodies. In such cases, cristae and limiting membranes were not easily identified (Figure 7 and inset). Mitochondria were mostly round and approximately 0.3 μ in diameter.

Postcalcitonin Treatment

Calcitonin treatment produced profound changes in the ultrastructure of osteocytes. One of the most striking changes occurred in the peri-

cellular space surrounding osteocytes. Following calcitonin therapy, practically all osteocytes examined were found embedded in a matrix of mature collagen (Figures 8 through 10). The osteocytes still exhibited a nucleus with peripheral condensation of chromatin and numerous central clumps of chromatin. However, after calcitonin treatment, there was an apparent increase in the amount of granular endoplasmic reticulum. The cytoplasm of most cells possessed an extensive rough-surfaced endoplasmic reticulum, the cisternae containing dense contents (Figures 8 through 10). Mitochondria were also more numerous. They no longer contained intramitochondrial microcrystal deposits. They all showed cristae and limiting membranes, and some contained typical intramitochondrial small dense granules (Figures 8 through 10). There was also a tremendous increase in cytoplasmic microfilaments and microtubules following calcitonin administration. Microfilaments often packed the cytoplasm of cell processes, sometimes appearing to be intimately associated with cell surface (Figures 8 and 9). Lysosome-like dense bodies were not abundant, but occasionally a residual body could be seen in the cytoplasm. The Golgi apparatus was well-developed. Overall, the cells exhibited a dense cytoplasmic texture, and occasionally pinocytotic vesicles were seen along the surface of cell processes (Figure 8).

Elastic Fibers

Treatment with calcitonin resulted in the appearance of large numbers of prominent extracellular elastic fiber-like structures scattered throughout the collagen matrix (Figures 8 through 10). Such structures, designated as elastic fibers as a result of their similarity to extracellular elastic fibers seen in connective tissue proper,[6] consist of large central amorphous cores surrounded by a peripheral aggregation of microfibrils (Figures 10, 11, and 14). When cut in cross-section, the microfibrils appeared tubular and had diameters that ranged from 10 to 15 nm (Figure 14). Also, when the elastic fibers were sectioned longitudinally, the 10- to 15-nm microfibrils did not exhibit apparent cross-banding as did the neighboring collagen fibrils (Figures 8 and 13).

A common finding in posttreatment bone was the presence of elastic fibers either adjacent to osteocytes or within an osteocytic cellular niche. For example, elastic fibers exhibiting small (Figure 8) to extremely large central amorphous core areas (Figures 10 through 12) were found within osteocytic cellular infoldings or closely apposed to the cell surface. Microfibrils forming an aggregate structure without any central amorphous core were occasionally seen within an osteocytic cell infolding (Figure 13). In contrast, examination of biopsy tissue taken to include the cartilaginous

cap revealed that cartilage, unlike bone, did not contain elastic fibers following calcitonin administration (Figure 15).

Discussion

The present ultrastructural observations add to our understanding of the cytopathology of familial bone dysplasia and also help define the possible intracellular mechanism by which calcitonin affects this disease process. The electron microscopic observations are consistent with earlier histologic findings and are also in agreement with current views of osteocyte structure and function. From the results of many histochemical studies performed during the past decade, a considerable amount of evidence has been obtained showing that osteocytes during their life cycle may have two functions: They appear to participate in both bone resorption [7-10] and bone formation.[11-14] Baud and Morganthaler [11,16] proposed that osteocytes may alternately resorb old and form new perilacunar matrix. This alternating activity has been termed "osteoplasis."

At the ultrastructural level, osteocytes in different phases of their activity cycle can be easily distinguished.[17-19] Those osteocytes which are resorbing bone contain little granular endoplasmic reticulum, few mitochondria, and variable numbers of lysosomes. They are typically found in large lacunae which are filled with a flocculent material instead of mature collagen fibrils. On the other hand, osteocytes which are forming bone matrix have an ultrastructure which is that of a cell active in protein synthesis for export. The osteocytes contain an extensive rough endoplasmic reticulum, a prominent Golgi apparatus, numerous mitochondria, and relatively few lysosomes. These cells are surrounded by a very small lacunar space and, thus, are in close apposition to the general bony matrix.

It is significant that before the administration of calcitonin osteocytes in the present case exhibited an ultrastructure almost identical to that of resorbing osteocytes previously described in studies of animals. The cells had minimal organellar development and, like the resorbing osteocytes of animals, were found in pericellular spaces in which bone had been lysed and seemed to be replaced with a flocculent material. The present ultrastructural observations are thus consistent with the view that osteocytic osteolysis is involved in the pathogenesis of familial bone dysplasia. Calcitonin treatment induced major changes in the ultrastructure of osteocytes of the diseased bone. Following calcitonin administration, the osteocytes changed to resemble those which have been found in animal studies to be in the bone-forming phase of their activity cycle. The cells developed an abundant granular endoplasmic reticulum, many mitochondria, an abundance of microfilaments, and many microtubules. They come to be surrounded by a matrix of mature collagen fibrils. Calcitonin

administration thus appears to have induced a functional change in the osteocyte population from a bone-lysing to a bone-forming phase. Certainly the ability to produce bone matrix by osteocytes at this time could account, in part, for the appearance of lamellar bone.

The present ultrastructural observations, however, also indicate that the osteocytes appear to be the cells solely responsible for the production of the elastic fibers found throughout the extracellular bone matrix following calcitonin administration. Electron microscopic studies of connective tissues, such as ligament and tendon, have shown that mature elastic fibers, ultrastructurally identical to the fibers in the present study, consist of two distinct morphologic components: a peripheral microfibrillar component and an inner, central amorphous core.[6,20-22] The amorphous core represents the largest component of the mature elastic fiber and has been identified as insoluble elastin.[6,23] Two stages have been recognized in the development of a mature elastic fiber.[6] In the first stage, fibers consist only of bundles of microfibrils and lack the amorphous component. During the second stage of development, the amorphous component gradually appears in the center of the fiber. As the elastic fiber reaches full maturity, the amorphous core comes to represent greater than 90% of the fiber. What is of major interest is that electron microscopic studies of connective tissue have shown that the connective tissue cell responsible for the synthesis of the proteins of the elastic fiber, the fibroblast, is closely associated both temporally and morphologically with elastic fiber development. Early developing fibers, consisting only of microfibrils, as well as maturing fibers are typically found aligned longitudinally within infoldings or hollows of the fibroblast surface. It has been proposed by Ross [6] and others that this intimate association between the cell surface of the fibroblast and developing elastic fibers determines the shape and direction of the forming elastic fiber. Therefore, it is of particular interest that in the present case osteocytes are the only cells which exhibit an intimate relationship with elastic fibers. Fibers in apparently different stages of development were found routinely in niches or infoldings of the cell surface of osteocytes.

It is well-established that elastic fibers are important constituents of tissues that contain elastomeric properties such as the wall of arteries, skin, and the alveolar septums of the lungs. However, the presence of elastic fibers either following calcitonin treatment or *de novo* within bone has not, to our knowledge, been previously reported. The possible function of elastic fibers in the present case, if any, is not known. It would be of interest to determine if long-term administration of calcitonin to normal animals can induce the formation of elastic fibers.

The present study also revealed that mitochondria of osteoblasts, osteo-

clasts, and osteocytes of dysplastic bone prior to calcitonin therapy often contained prominent microcrystalline inclusion bodies. A similar observation had been made earlier in two other children with this disease process.[24] In this regard, it is interesting that in a recent study of the ultrastructure of bone of uremic patients with renal osteodystrophy Bonucci and Gherardi noted different types of osteocytes.[25] One type of osteocyte, designated resorptive osteocytes, like the osteocytes of the diseased children in the present case, uniquely contained intramitochondrial accumulations of inorganic crystals. Similar intramitochondrial crystals have also been seen in human skeletal muscles in a number of neuromuscular disorders [26,27] and in various experimental pathologic conditions. They have been found in rabbit aorta after adrenalin administration [28] and in the myocardium of animals given corticosteroids,[29] plasmocid,[30] low doses of isoproterenol,[31] or carbon tetrachloride.[32] From the results of electron diffraction studies, such intramitochondrial microcrystal deposits are believed to be calcium.[28] Mitochondria play a major role in intracellular calcium regulation in many cells [33] and it seems reasonable to suppose that the deposits in diseased bone cells are a consequence of increased cell activity in removing bone.

The precise pathologic significance of the mitochondrial calcification of osteoblasts, osteoclasts, and osteocytes of patients with familial bone dysplasia must await further study. Nevertheless, it is interesting that calcitonin administration resulted in the disappearance of microcrystals from mitochondria of osteocytes and osteoclasts but not in those of osteoblasts. In fact, calcitonin had no apparent effect on osteoblast structure at all. This latter observation is consistent with the view that calcitonin does not enhance bone formation by osteoblasts.[34] It is unlikely that the calcitonin effect on mitochondria of osteocytes is a direct one, since biochemical studies suggest that polypeptide hormones do not enter cells. It is more reasonable to assume that the mitochondrial change results from an interaction between calcitonin and a specific receptor site located on the plasma membrane of the osteocyte with a consequent reduction in the enhanced transcellular calcium flux engendered by osteocytic osteolysis. Regardless of how the mitochondrial change occurs, the response of osteocytic mitochondria, in contrast to that of osteoblasts, is consistent with our view that, at least in the present case, the primary target cell for calcitonin action in bone is the osteocyte. The sparcity of osteoclasts in this disease process strongly indicates that surface osteoclasia plays little or no role in the pathogenesis of familial bone dysplasia. Thus, this childhood disease seems quite different from adult Paget's disease, in which involvement of osteoclasts is prominent.[35] It is also of interest that

the nuclei of the few osteoclasts encountered in our specimens did not exhibit any nuclear inclusions. It has been suggested that the presence of particles in the nuclei of osteoclasts of adult Paget's disease may be related to the pathogenesis of the disease.[35] In addition, the osteoclasts of the patients with hereditary bone dysplasia which we examined contained only two or three nuclei per cell. This is unlike adult Paget's disease, in which osteoclasts contain large numbers of nuclei per cell.[35]

We have complied with all the requirements outlined by the Code of Ethics of the World Medical Association of Human Experimentation. Consent was properly obtained from both parents of the children for both tests and treatment after full explanation of the purpose and scope of all procedures. Any potential hazards or side effects were completely explained. The entire protocol of the investigation and therapy was approved by The Human Rights Committee of Cornell University Medical College.

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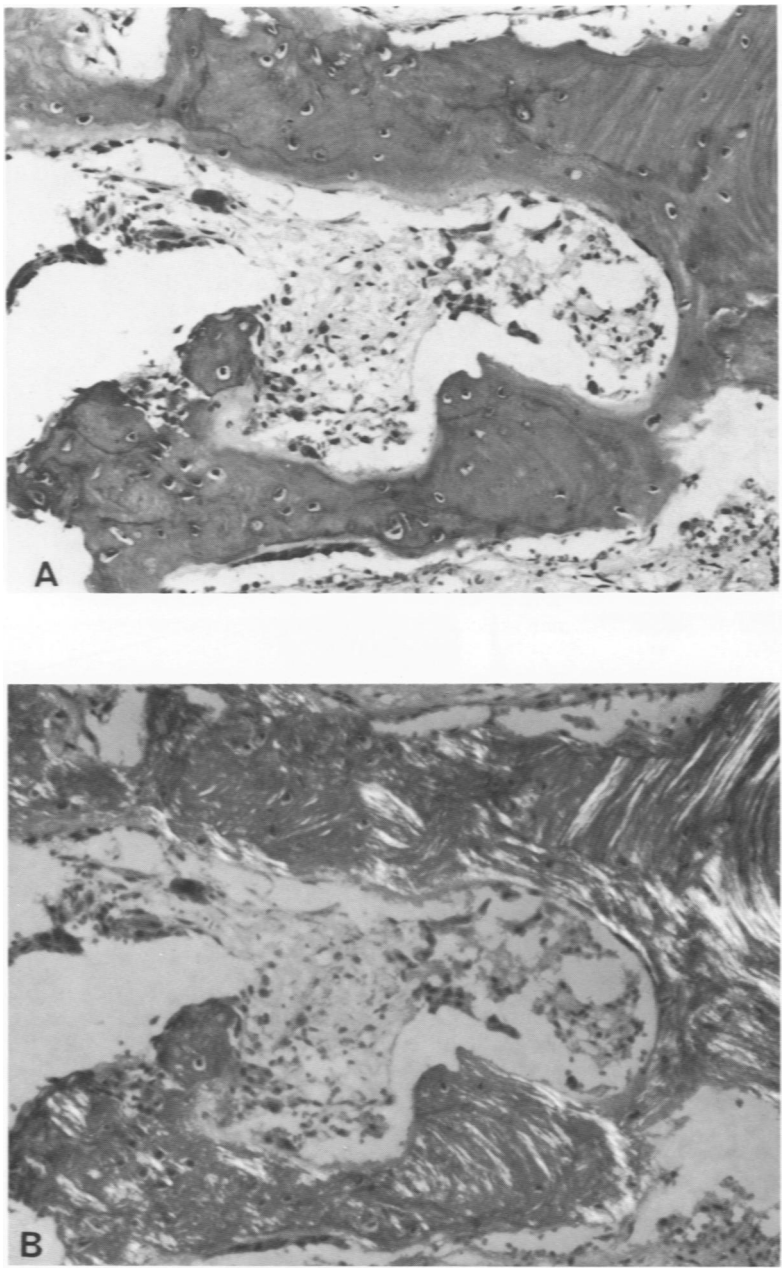
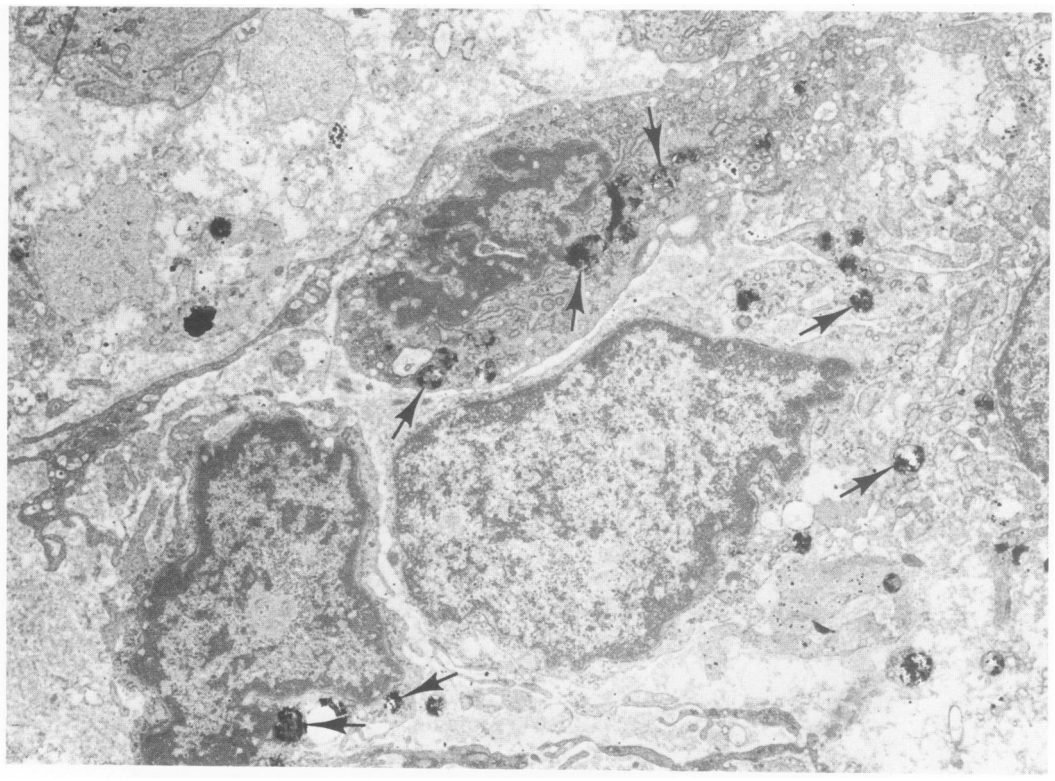


Figure 1A—Section showing apophyseal slender trabecula of affected girl. Histologically, the section reveals immature woven bone characterized by a highly intense osteocytic osteolysis, no cartilaginous core, and wide osteoid seams. Note the loose connective tissue between trabeculae. **B**—Same field as A, exposed with polarized light, demonstrating very poor birefringency of immature collagen. (H&E, ×180)



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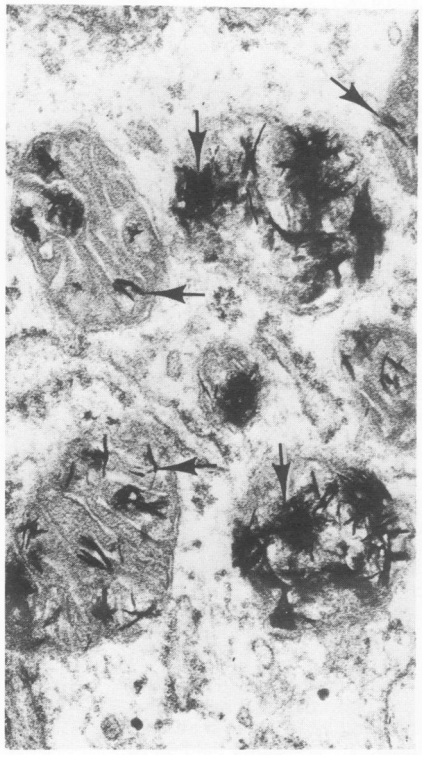

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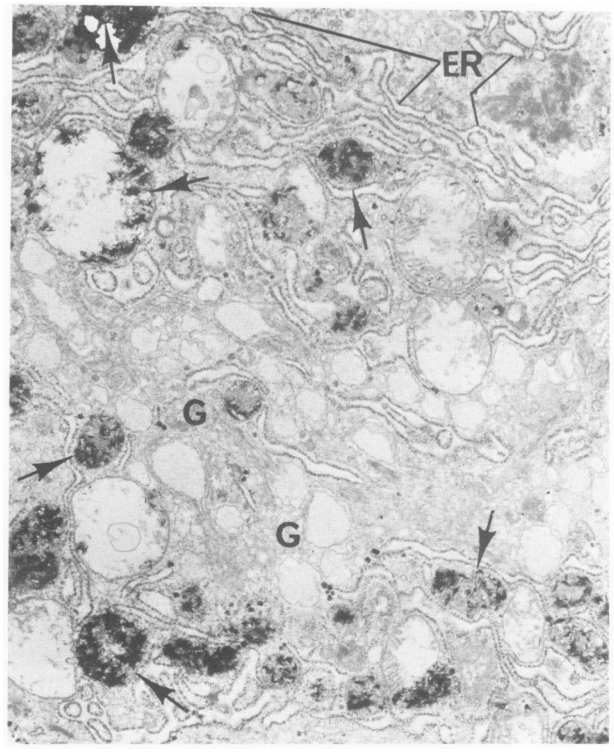

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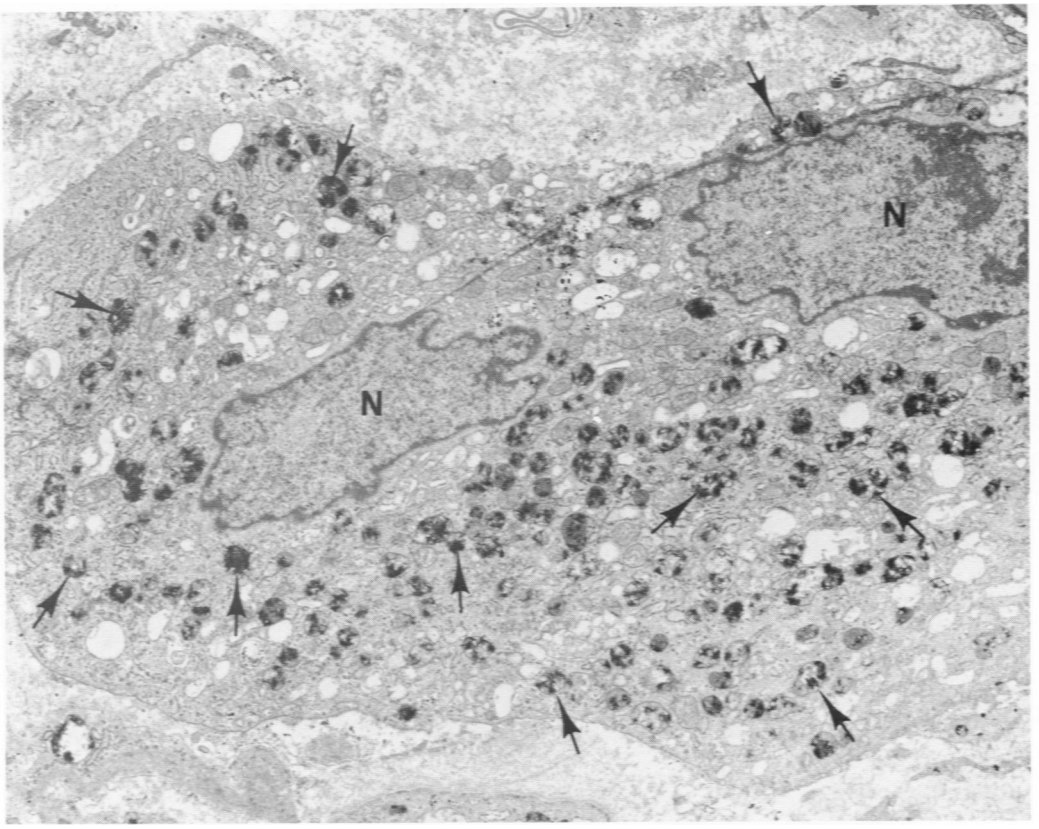
Figure 2—Electron micrograph of a group of osteoblasts from diseased child (BL-30) before calcitonin treatment. The cells are characterized by the appearance of clumps of dense microcrystals in the matrix of mitochondria (*arrows*). (×8000) **Figure 3**—Electron micrograph of a portion of an osteoblast from bone of diseased patient (MA-31). Note the needle-shaped microcrystalline inclusion bodies in the mitochondria (*arrows*).(×40,500) **Figure 4**—Electron micrograph of osteoblast from patient (MA-31) after 6 months of calcitonin treatment. The osteoblast contains a well-developed Golgi complex (*G*) and rough-surfaced endoplasmic reticulum (*ER*). The majority of mitochondria (*arrows*) still contain dense crystalline masses. (×13,400)



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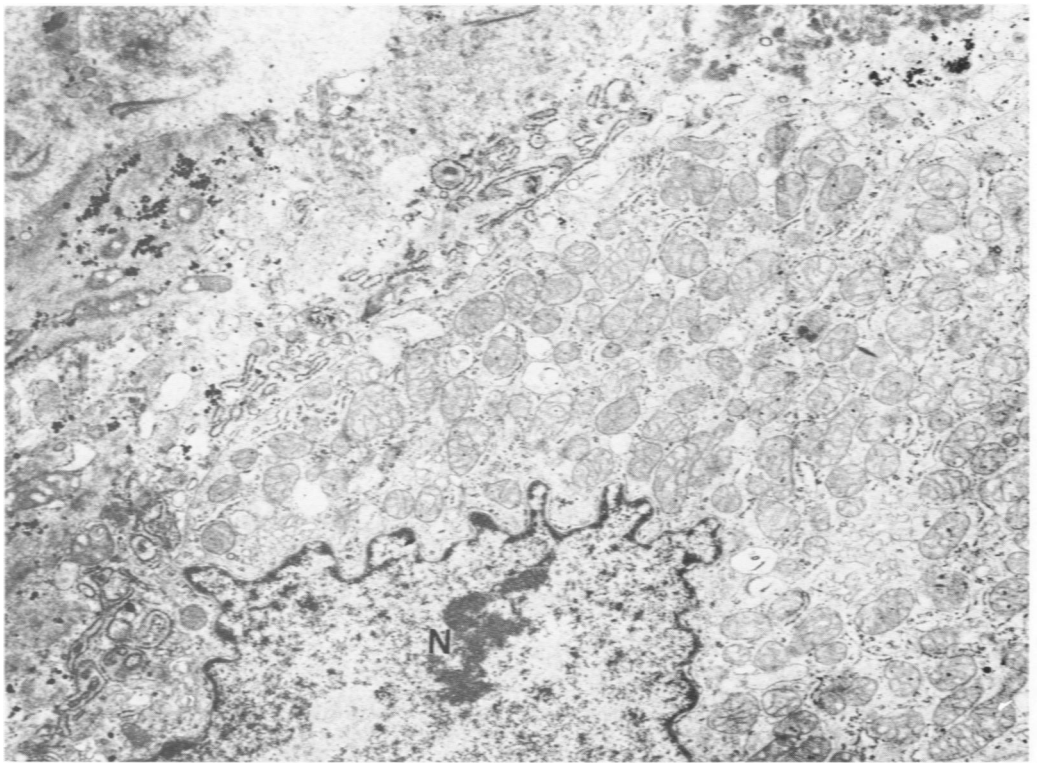

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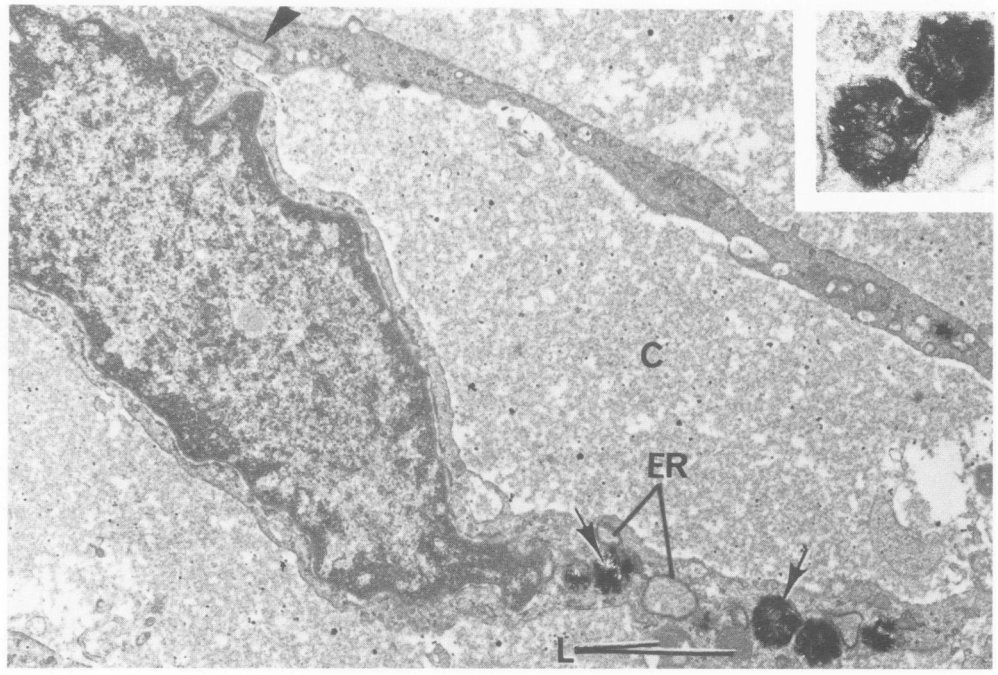
Figure 5—Electron micrograph of an osteoclast prior to treatment. The cell is distinguished by large numbers of mitochondria which fill the cytoplasmic matrix. The majority of mitochondria (*arrows*) contain dense microcrystal deposits. Two normal-appearing nuclei (*N*) are also illustrated. (×5600) **Figure 6**—Electron micrograph of an osteoclast after 4.5 months of treatment with calcitonin. Mitochondria lack the microcrystalline deposits. No other major ultrastructure changes occurred during hormonal administration. *N* = nucleus. (×10,500)



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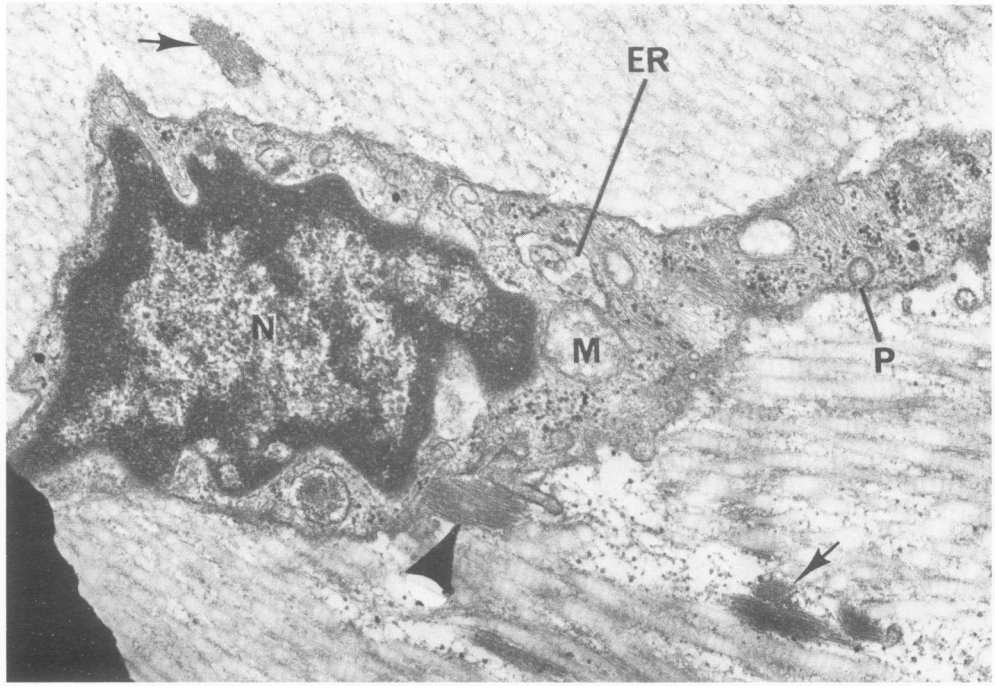

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Figure 7—Osteocyte from diseased bone (MA-30) prior to calcitonin therapy. Minimal organellar development is found in the cytoplasm. A characteristic feature of osteocytes is that mitochondria, usually few in number, are heavily loaded with intramitochondrial microcrystalline bodies which make mitochondria appear as solid dense bodies (*arrows*). Also seen in the micrographs are profiles of the granular endoplasmic reticulum (*ER*) and several lysosome-like bodies (*L*). Note that the wide pericellular space consists primarily of a flocculent material (*C*). Also note the cell-to-cell specialization between cytoplasmic strands of adjacent osteocytes (*arrowhead*). (×12,000; **insert**, ×24,750) **Figure 8**—Electron micrograph of an osteocyte after 4.5 months of calcitonin treatment. The osteocyte is surrounded by a matrix of regularly oriented collagen fibrils. Note the presence of several aggregates of microfibrils (*arrows*) in the extracellular matrix, one of which is closely apposed to the cell surface (*arrowhead*). Microfilaments and microtubules are abundant throughout the osteocyte cytoplasmic matrix. Mitochondria (*M*), nucleus (*N*), pinocytotic vesicle (*P*), and granular endoplasmic reticulum (*ER*) are also shown. (×22,700)

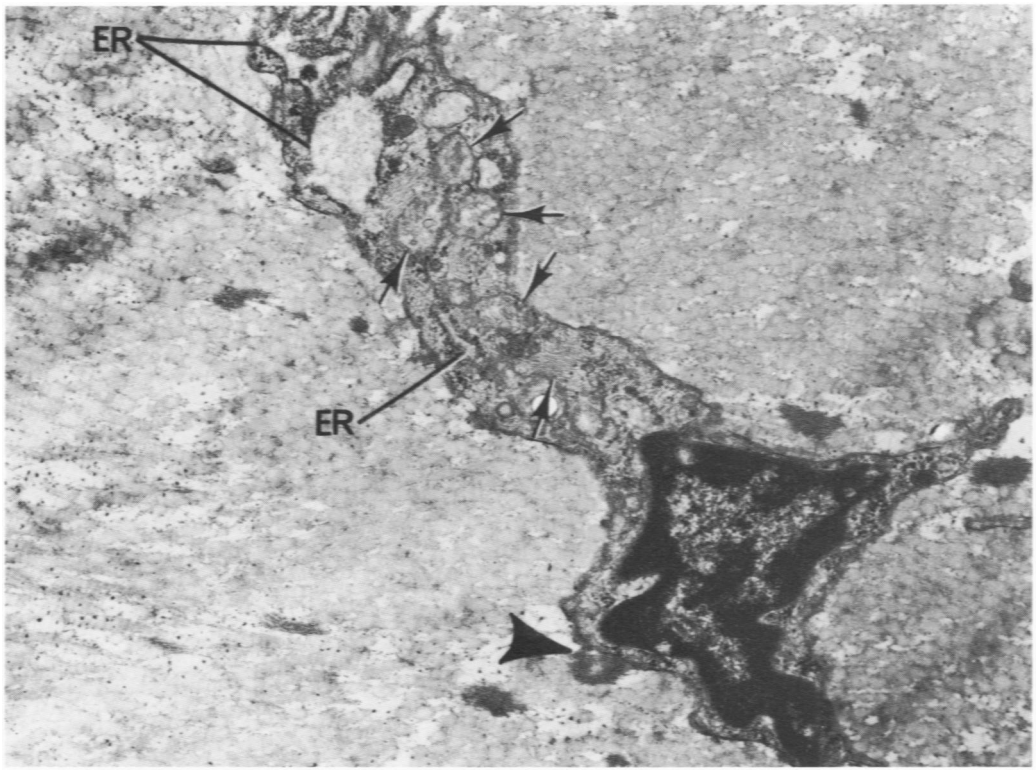


Figure 9—Electron micrograph of an osteocyte and surrounding bone matrix from diseased patient (MA-31) after 6 months of calcitonin treatment. The osteocyte exhibits a well-developed granular endoplasmic reticulum (*ER*). The cisternae of the endoplasmic reticulum contains a dense material, and the reticulum is heavily dotted with ribosomes. Mitochondria are numerous (*arrows*) and do not contain the dense microcrystalline inclusion bodies. Note that the cell is embedded in a matrix of normal collagen fibrils. *Arrowhead* points to an elastic fiber-like structure containing a small central amorphous area which is closely apposed to the cell surface. (×15,750)

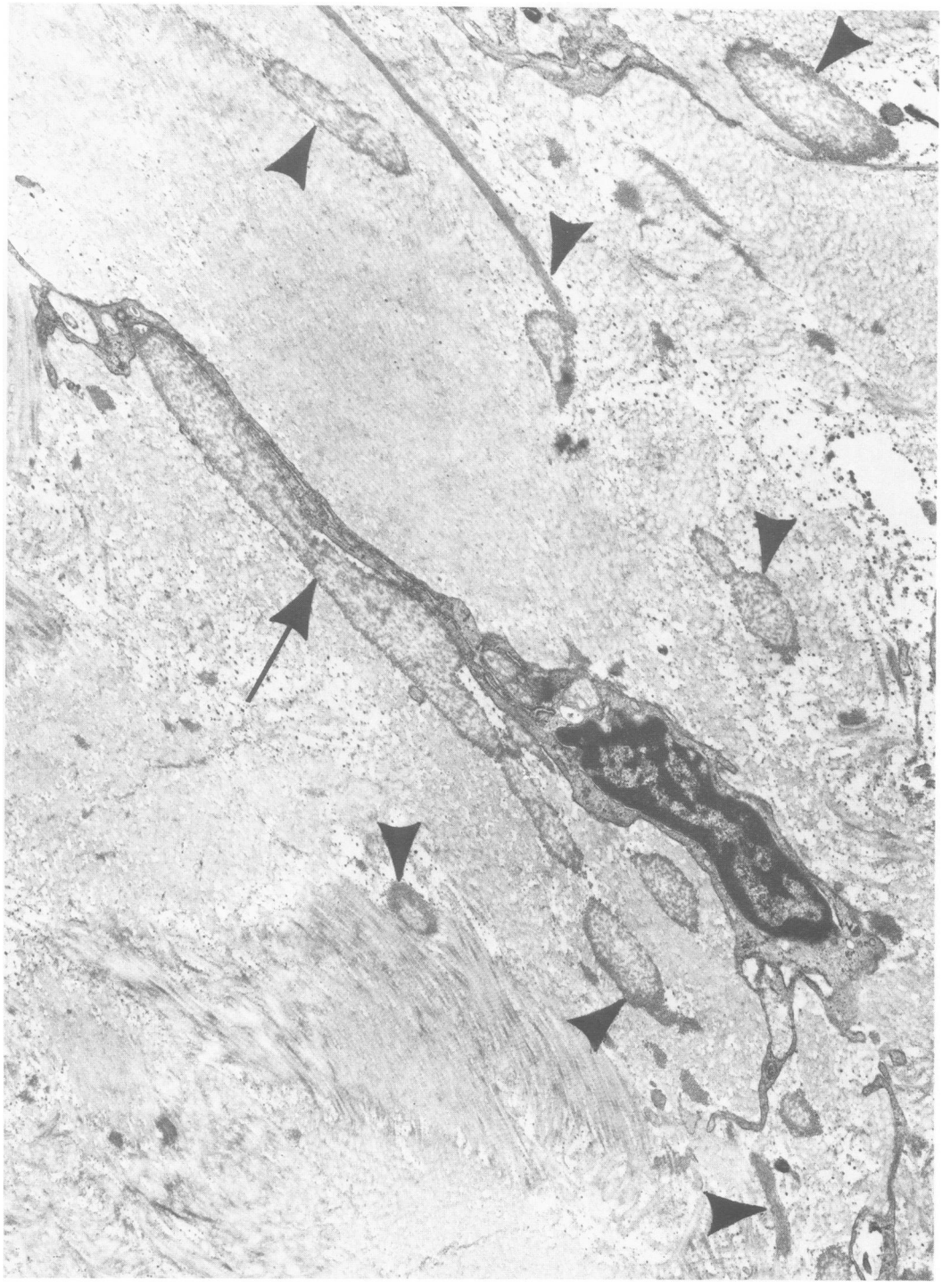
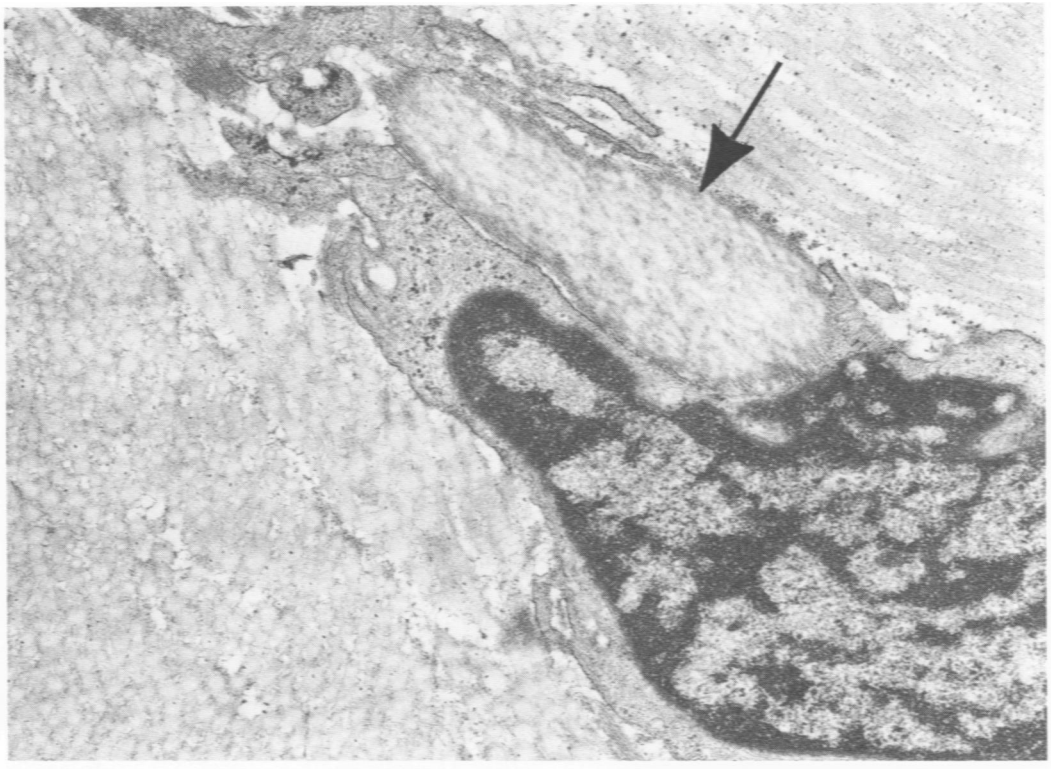


Figure 10—Electron micrograph of iliac crest 6 months after treatment with calcitonin. The extracellular matrix contains numerous prominent elastic fiber-like structures (*arrowheads*). Such structures, like elastic fibers of connective tissue, contain a large central amorphous core and an envelope of peripheral microfibrils. Note the close association between the elastic fiber (*arrow*) and the osteocyte in the center of the field. Collagen fibrils sectioned both longitudinally and transversally can also be seen. (×10,750)



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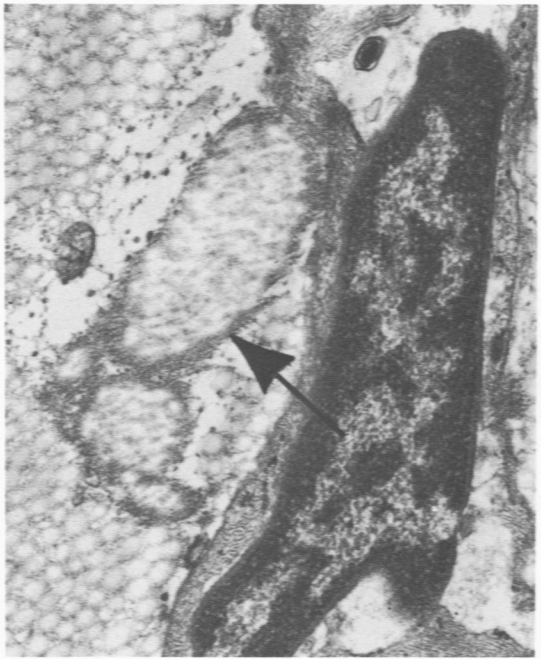

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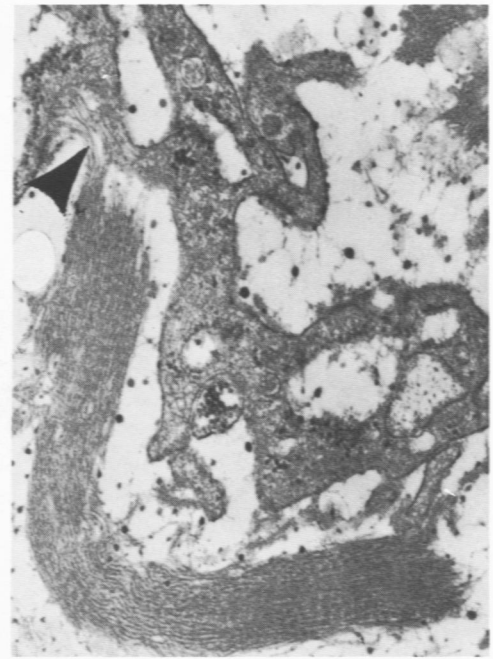

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Figures 11 and 12—Electron micrographs illustrating the close association between elastic fibers (*arrows*) and deep-seated osteocytes characteristic of bone treated with calcitonin. (**11**, ×18,700; **12**, ×25,800) **Figure 13**—Electron micrograph illustrating an aggregate of microfibrils in an osteocytic cellular niche (*arrowhead*). The microfibrils do not as yet contain a central amorphous core. Calcitonin-treated bone, 6 months. (×27,000)

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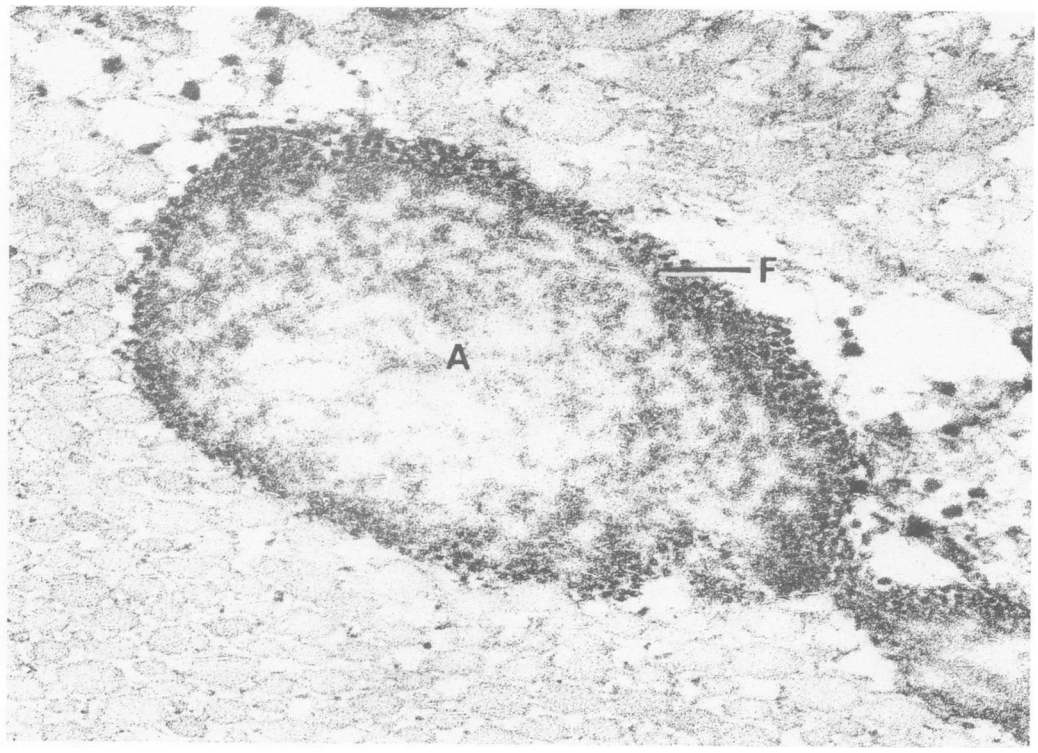

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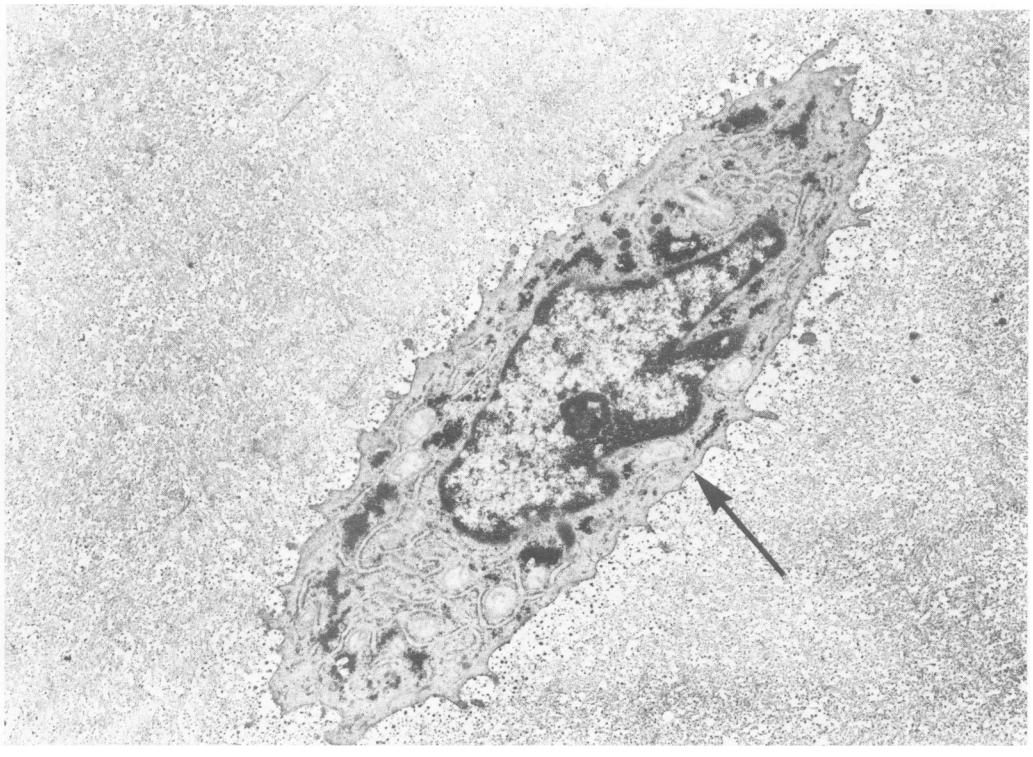

Figure 14—High magnification of an elastic fiber from bone treated with calcitonin. The peripheral microfibrils (*F*) are in cross-section, appear tubular, and have diameters of 10 to 15 nm. The central amorphous core (*A*) occupies nearly 90% of the volume of the elastic fiber. (×67,800) **Figure 15**—Electron micrograph of cartilage after 1 year of treatment with calcitonin. The chondrocyte (*arrow*) and the extracellular matrix are normal in appearance. Elastic fibers were not found. (×10,500)