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Study on Normal and Otosclerotic Bone Cell Cultures:

An Advance in Understanding the Pathogenesis of Otosclerosis

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The authors first reviewed the main theories concerning the pathogenesis of otosclerosis and studied the morphologic and functional characteristics of cell cultures derived from normal and otosclerotic bones. Light transmission and scanning electron microscopy did not permit definite identification of the cultured cells as predominantly osteoblasts, nor did these techniques show significant differences between cultured cells derived from normal and pathologic bone. Functional tests of the cell cultures proved more interesting. First, the bony nature of the cultured cells was demonstrated by studying the intracellular $^{45}Ca^{++}$ uptake after stimulation with calcitonin and dybutryl-cAMP. Second, cell cultures derived from otosclerotic bone behaved differently from those derived from normal bone. Their peak uptake of calcium appeared later, and post-stimulatory values were higher, suggesting that cells derived from otosclerotic bone store a greater quantity of $^{45}Ca^{++}$. Furthermore, after stimulation with calcitonin and propranolol, we observed an inhibition of the calcium uptake and decreased intracellular cAMP levels in normal bone cell cultures. In contrast, the cell cultures derived from otosclerotic bone exhibited an initial inhibition of calcium absorption followed by massive calcium penetration.

The response of adenylate cyclase to the action of Mg^{++}, Ca^{++}, and F^- ions was evaluated in cultures derived from normal bone, otosclerotic bone, and normal skin fibroblasts. The resulting data show that activation due to Mg^{++} is much lower in cultured cells derived from otosclerotic bone than in those from either normal bone or skin fibroblasts. No significant differences were found after Ca^{++} inhibition in any of the cell cultures. Moreover, in cell cultures derived from normal bone, F^- ions induced a strong activation that was lower than the levels observed in cultures of otosclerotic bone or in normal fibroblasts. We hypothesize that an alteration at the calcitonin receptor site is responsible for the difference in calcium uptake and cAMP levels observed in the cells derived from otosclerotic bone as compared to those cultured from normal cells.

HISTORY

Stapedo-ovalar ankylosis was first observed by Valsalva in 1741,[1] and the labyrinthine capsule involvement during otosclerosis was described by Politzer in 1893. The relevant literature offers five major mechanisms for the etiology of otosclerosis:

1. Otosclerosis as a localized manifestation of degenerative arthritis,[3,4] resulting from mechanical stress,[4–6] hormonal factors and genetic predisposition,[2,3,7–18] osteochondropathies,[19–22] or irradiation[23]
2. Otosclerosis as a result of vascular defects[24]
3. Otosclerosis as a result of bone cell dysfunction[2,17,25–44]
4. The enzymatic theory of the etiology of otosclerosis[35,45,46]
5. Otosclerosis as an autoimmune disorder.[47–52]

Although some of these proposals have merit, none has convincingly been shown to comport with the true nature of the pathogenesis of otosclerosis.

At the outset of our research, one crucial question remained unanswered: How does oto-

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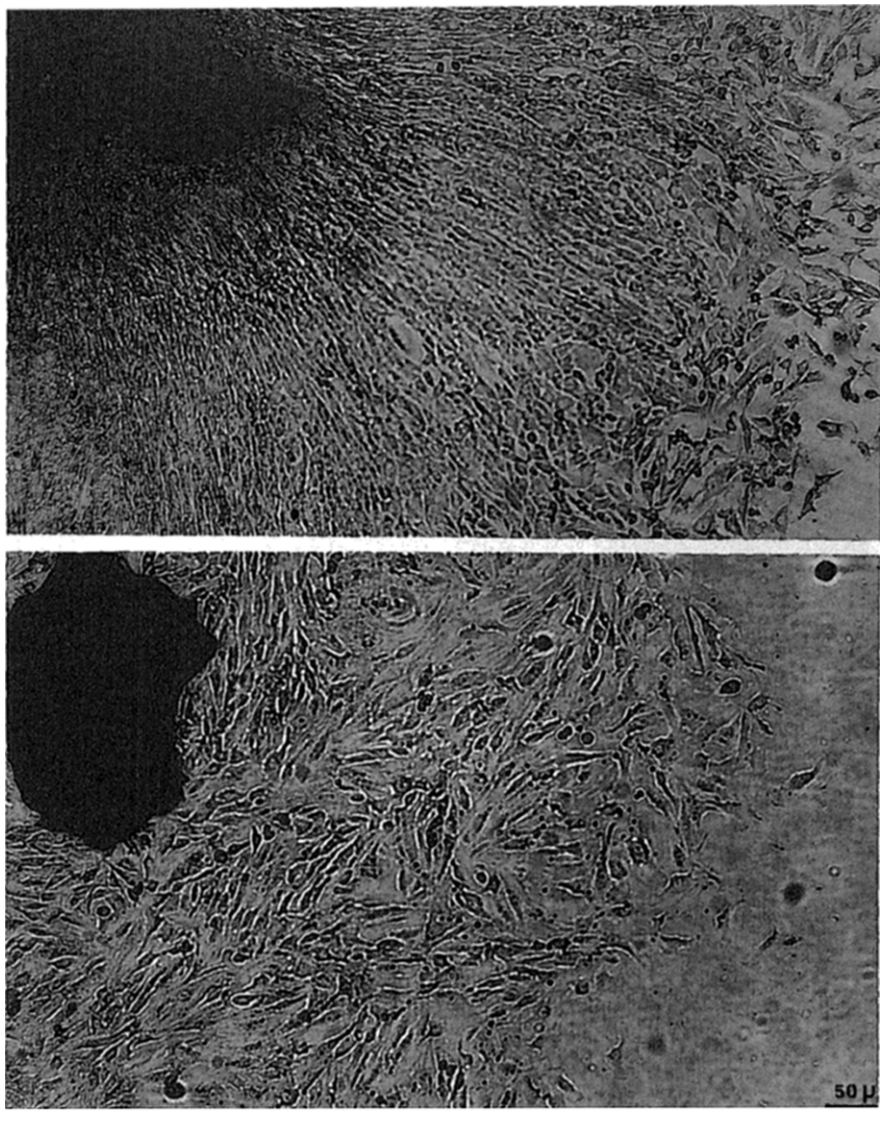

Figure 1. Outgrowth of cells surrounding temporal bone fragments at 7–10 days of culture. (Reproduced with permission.[56])

sclerosis begin? Of the morphologic findings, the studies on the mineral content of the otosclerotic bone, even the enzymatic theory, none reflects the cause, but rather the results, of a pathologic event. What we do know is that for some reason bone cells do not function normally. They rupture, delivering lysosomal enzymes that, together with activated osteoclasts, start the osteolysis of the surrounding bone tissue.

Our purpose was to study bone cells, intensively, observing their main functions to investigate not only some aspects of their metabolism, such as calcium incorporation, but also some characteristics of their cell membranes. A further area for investigation is suggested by the established links between otosclerosis and certain hormonal events. An investigation of receptor sights might involve exploring the cellular membrane to various hormones.

The remaining problem involved securing a sufficient number of bone cells. The only available source was a culture of very small fragments of otosclerotic bone collected during surgery. The cultured cells can then be compared with normal bone cells obtained from the crura and the bone of the external meatus.

We began by choosing a bone with the smallest possible amount of marrow to minimize the growth, in culture, of non-bone cells such as fibroblasts. The head and neck of the stapes contain marrow. The crura consist of semicylindrical shells of cortical bone. The footplate, in contrast, arises embryologically from the otic capsule. We were confident that cell cultures of such specimens could provide sufficient material for our experiments.

MATERIALS AND METHODS

The otosclerotic bone specimens were carefully selected intraoperatively, by microscopic

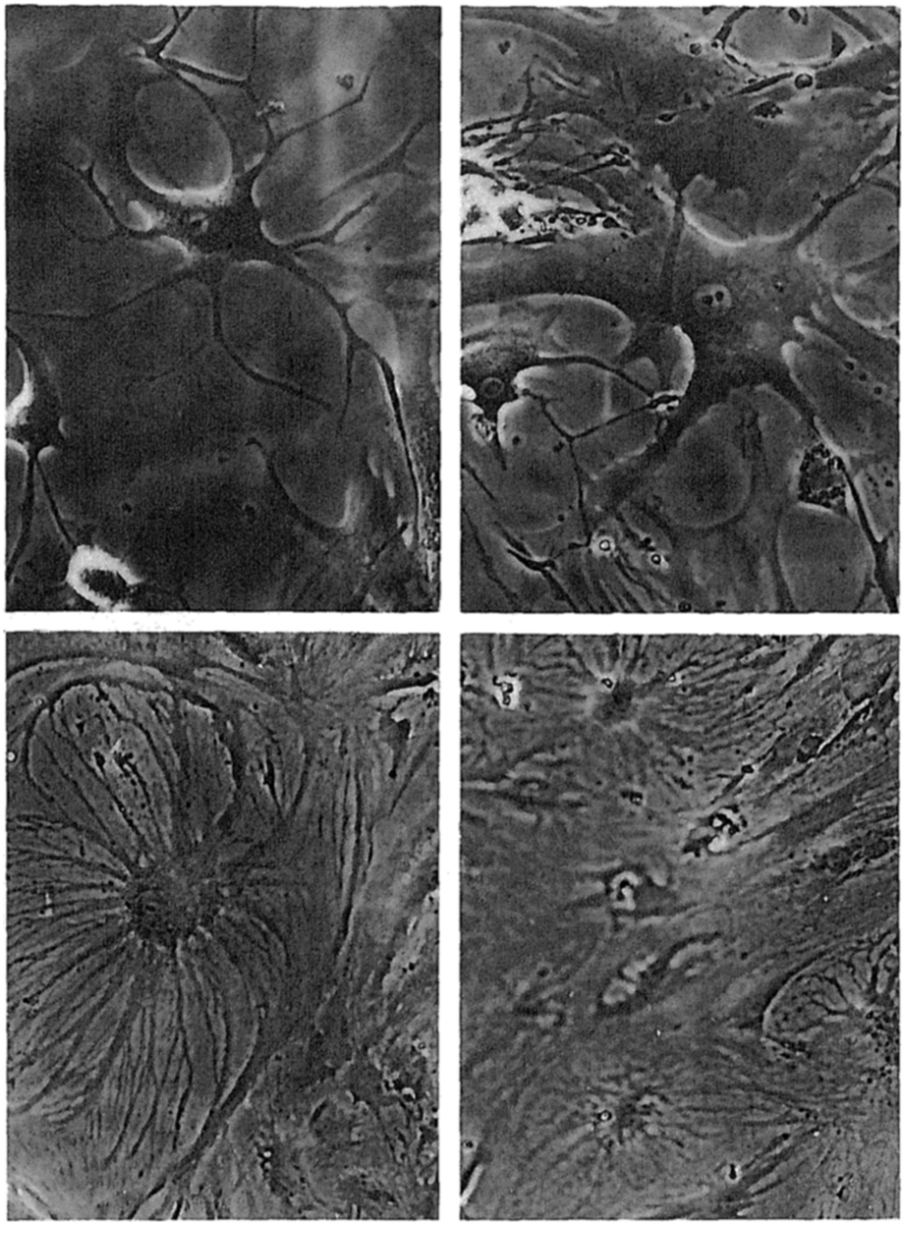

Figure 2. *Top left and right*, different types of bone-derived cells in non-confluent monolayers and confluent monolayers (*bottom left and right*). (Reproduced with permission.[56])

examination, and consisted largely of fragments of the footplate fixed within the oval window. In some cases, the foci were involving the promontory. The stapes superstructure was obtained during surgery.

Specimens were immediately stored separately in minimal essential medium containing 20% foetal calf serum, essential aminoacids, vitamins, and antibiotics.

The living cells were then isolated from bone matrices. Methods described in the literature use enzymes to digest the matrix and liberate the cells contained therein.[53,54] Unfortunately, bone samples taken during the stapedectomies were too small to permit the use of this technique that could have damaged the cells and caused excessive loss. The only previous report that has come to our attention on the culture of otosclerotic bone fragments describes a chicken plasma clot method that makes it difficult to obtain free cells for use in subcultures.[5,55] Therefore, a thin layer culture, generally used to induce cell separation in various tissues, seemed to be the most promising method for our purpose. Using their technique as previously described,[56] tiny tissue fragments adhering to the surface of culture flasks can be supported with a minimum of culture medium. Like other tissues, the bone biopsies gave rise to an outgrowth of cells surrounding the spontaneously disintegrating fragments within a period of 7 to 15 days (Fig. 1). When the outgrowth around the fragment

reached a diameter of 2 to 3 cm, subcultures were prepared by detaching the monolayer by trypsin: EDTA treatment.

The cells that grew out from the cultured bone fragments appeared to be of four types: 1) osteoblast-like cells with large body and cytoplasmatic processes, 2) fibroblast-like spindle shaped cells, 3) cells intermediate between these two types, and 4) osteoclast-like multinucleated cells (Figs. 2, 3). All cell types were detectable in cultures of otosclerotic footplate and of normal bone fragments derived from the external auditory meatus of the same patient. The first three types, moreover, were also observed in secondary cultures, whereas the osteoclast-like cells were much rarer and present only in primary cultures.

Further characterization of the cell cultures was achieved using scanning and transmission electron microscopy.[57] Two types of cells were identified by scanning electron microscopy: star-shaped cells with cytoplasmatic processes and microvilli (Fig. 4) and smooth, spindle-shaped cells (Fig. 5). Transmission electron microscopy revealed the same two cell types. One was an irregular osteoblast-like cell with a large body, well-developed endoplasmic reticulum, electron-dense cytoplasmic granules, and a multilobulated nucleus (Fig. 6). The other was a spindle-shaped fibroblast-like cell with elongated nucleus and homogeneous cytoplasm (Fig. 7). No difference was observed, however, between cells cultured from normal and from pathologic bone samples.

Because of the suggested mechanisms for the pathogenesis of genetic alteration in osteoblastic activity, we analyzed the karyotype of both otosclerotic and normal bone cells. Thirty metaphases each of normal and pathologic bone cultures were scored for each of the patients to detect constitutional or acquired chromosomal abnormalities. G-banding karyotype analysis (Fig. 8) was performed according to the trypsin-Giemsa technique[58] to better recognize both structural and numeric cytogenetic aberrations. This technique revealed that both normal and pathologic bone cells have a completely normal karyotype; the former were taken to be the patient's constitutional karyotype. This finding weakens the hypothesis that this disease is associated with a specific chromosomal alteration.

It has been demonstrated that the morphology of cultured bone-derived cells is variable, and that even the same cell may change shape spontaneously or in response to exogenous stimuli.[59]

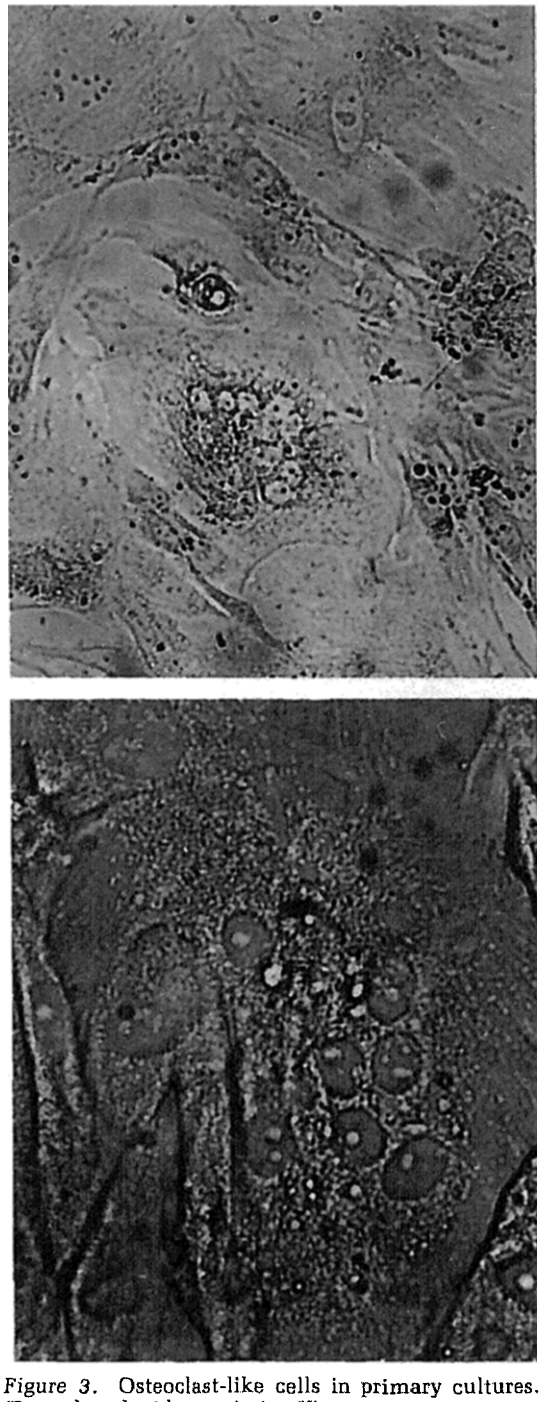

Figure 3. Osteoclast-like cells in primary cultures. (Reproduced with permission.[56])

Therefore, characterization of cultured cells based on morphologic features alone is unreliable. On the basis of the knowledge that, even in culture, some hormones such as calcitonin (CT) and parathyroid hormone (PTH) can stimulate the uptake of Ca^{++} ions,[54] we attempted to functionally characterize normal and otosclerotic cells by evaluating $^{45}Ca^{++}$ incorporation after calcitonin stimulation.

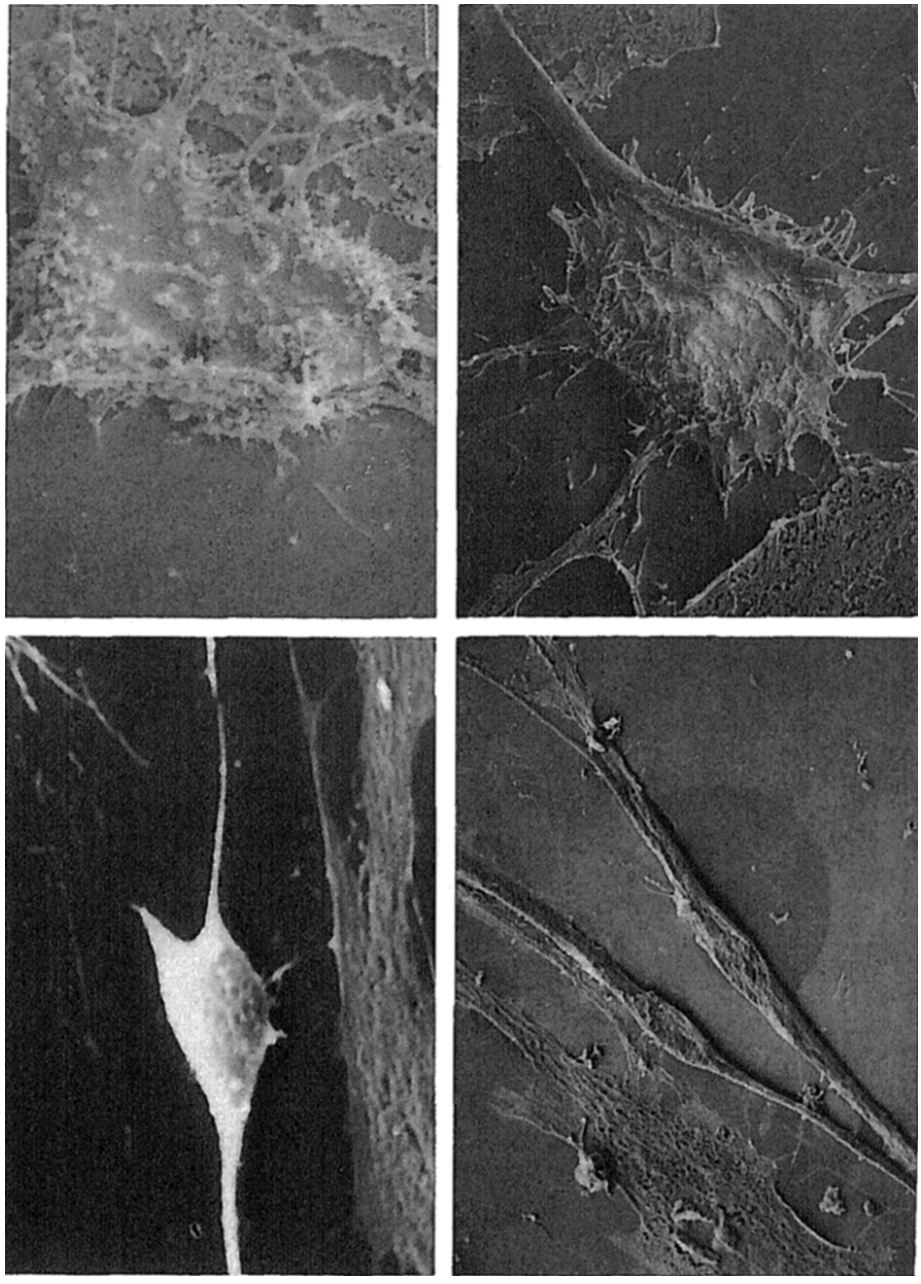

Figure 4. (*top*). Scanning electron microphotographs showing osteoblast-like cells with several cytoplasmic processes and microvilli. (Original magnification, ×5000.) (Reproduced with permission.[57])

Figure 5. (*bottom*). Scanning electron microphotographs of spindle-shaped and smooth-surfaced fibroblast-like cells. (Original magnification; left, ×1250; right ×1000.) (Reproduced with permission.[57])

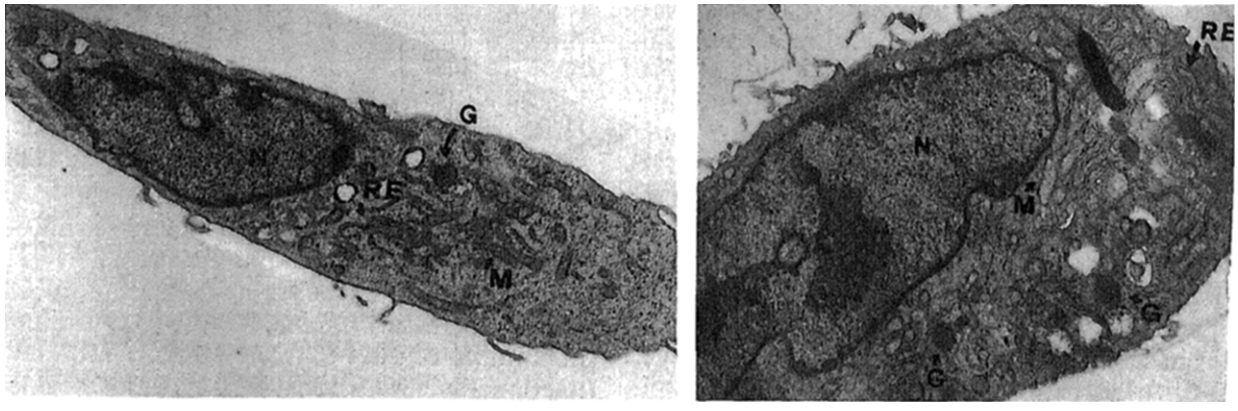

Figure 6. Transmission electron microphotographs of osteoblast-like cells. The nucleus is characteristically peripheral and multilobulated; the cytoplasm contains several mitochondria (M), an abundant endoplasmic reticulum (RE) with enlarged cisternae, and electron-dense granules (G). (Original magnification; *left*, ×11,500; *right*, ×12,600.) (Reproduced with permission.[57])

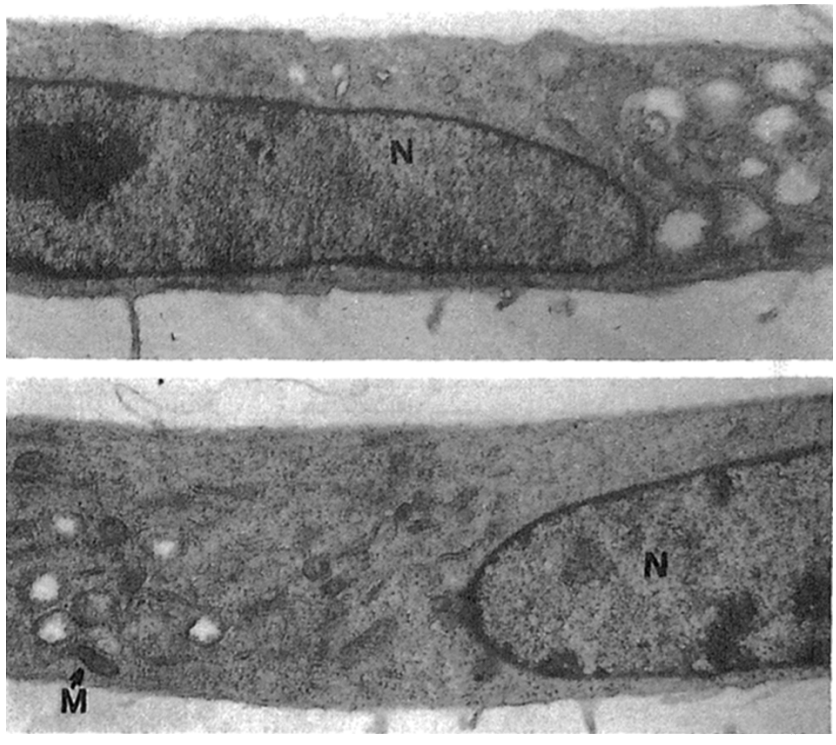

Figure 7. Transmission electron microphotographs of spindle-shaped fibroblast-like cells with elongated nucleus (N) and homogeneous cytoplasm containing several mitochondria (M). (Original magnification; *top*, ×14,000; *bottom*, ×12,600.) (Reproduced with permission.[57])

The first series of experiments was performed on cell cultures obtained from normal bone biopsies of 5 different patients. We noted a substantial difference between normal bone-derived cells and the skin fibroblasts used as controls (Fig. 9). Ca^{++} uptake was low in CT treated fibroblasts and virtually identical to the controls of untreated fibroblasts and bone-derived cells. Calcium incorporation was clearly increased in CT-stimulated (0.5 IU/ml) bone-derived cells. A lower dose of 0.1 IU/ml did not produce any noteworthy variation in $^{45}Ca^{++}$ incorporation, and higher doses of 1.0 and 2.0 IU/ml did not change the reaction either qualitatively or quantitatively (Fig. 10). The intracellular Ca^{++} flow induced by CT stimulation was greatly enhanced by the influence of an electromagnetic field (Donti et al., unpublished data (Fig. 11).

Because the dybutyryl-derivative of cylic adenosynmonophosphate (db-cAMP) mimics hormonal action by facilitating the penetration of Ca^{++} into isolated bone-derived cells,[60,61] we used it to treat normal bone-derived cell cultures.[56] The resulting calcium uptake was sub-

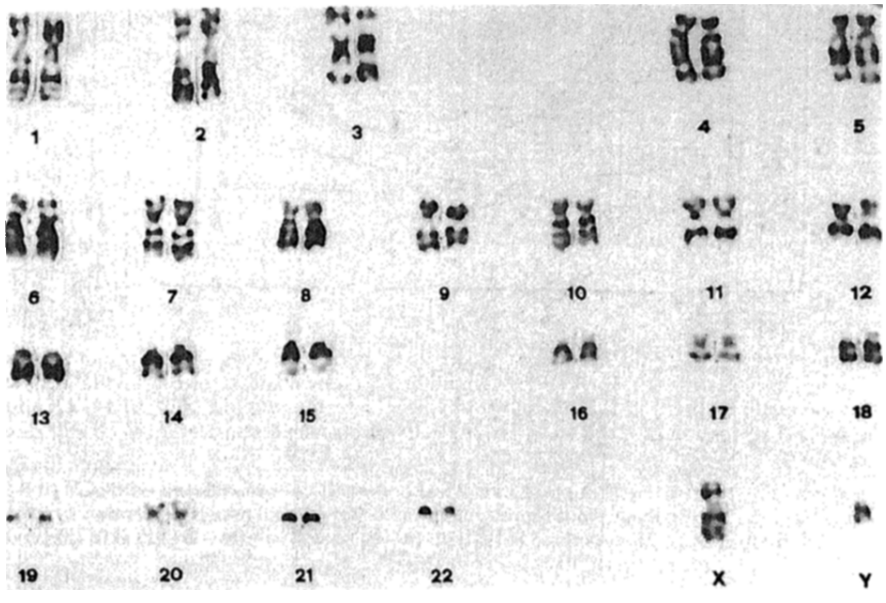

Figure 8. Normal 46,XY G-banded karyotype of cells obtained from otosclerotic bone.

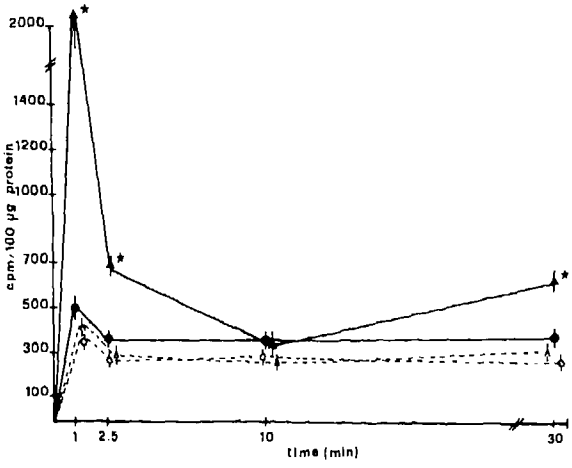

Figure 9. Effect of sCT (0.5 I.U./ml) on $^{45}Ca^{++}$ uptake in bone-derived cell cultures (▲—▲) and skin-derived fibroblast cultures (△--- △). The circles (● and ○) represent the calcium uptake in the same two cell populations incubated without sCT. Each point represents the mean ± SE (n = 8). Statistical evaluation compares these data with those obtained from cells incubated with $^{45}Ca^{++}$ alone ($p < 0.01$). The upper curve shows the results of experiments performed on a single bone biopsy, because the other 4 samples displayed similar patterns of response but different absolute values of radioactivity. A significant increase in calcium uptake following hormonal stimulation was observed only in bone-derived cell cultures. (Reproduced with permission.[58])

stantially the same as that after calcitonin stimulation (Fig. 12) with one exception. The period for maximum uptake was longer, probably because the derivative nucleotide is slower to penetrate the cells.

A second series of experiments on otosclerotic bone-derived cells used only normal bone-derived cells as controls (Fig. 13). The two cell populations exhibited only a slight difference in the time required for $^{45}Ca^{++}$ to reach maximum incorporation (2.5 minutes in pathologic and 1 minute in normal cells). Although this research yielded reliable evidence that cultured cells were indeed bone cells, it failed to reveal any difference between normal and otosclerotic bone-derived cells.

Distinctive aspects started to emerge when CT stimulation was performed in the presence of propranolol. Experimental data suggest that β receptors mediate the action of calcitonin on target cells.[62,63] To test this hypothesis and better characterize the CT-receptors on otosclerotic bone-derived cells, we used propranolol on normal bone-derived cultures. This experiment led to the discovery that the two cell cultures behaved differently. Propranolol caused a stable inhibition of the intracellular uptake of $^{45}Ca^{++}$ in normal bone-derived cells, and in otosclerotic cells it provoked a transitory inhibition followed by a massive penetration of Ca^{++} ions (Fig. 13). These results indicate a difference between normal and pathologic tissues and support the validity of our approach to studying the pathogenic mechanism of otosclerosis.

cAMP is assumed to be the intracellular messenger of the CT-induced signal.[64,65] Measured levels of cAMP revealed similar responses in the two cell types after CT and CT + propranolol

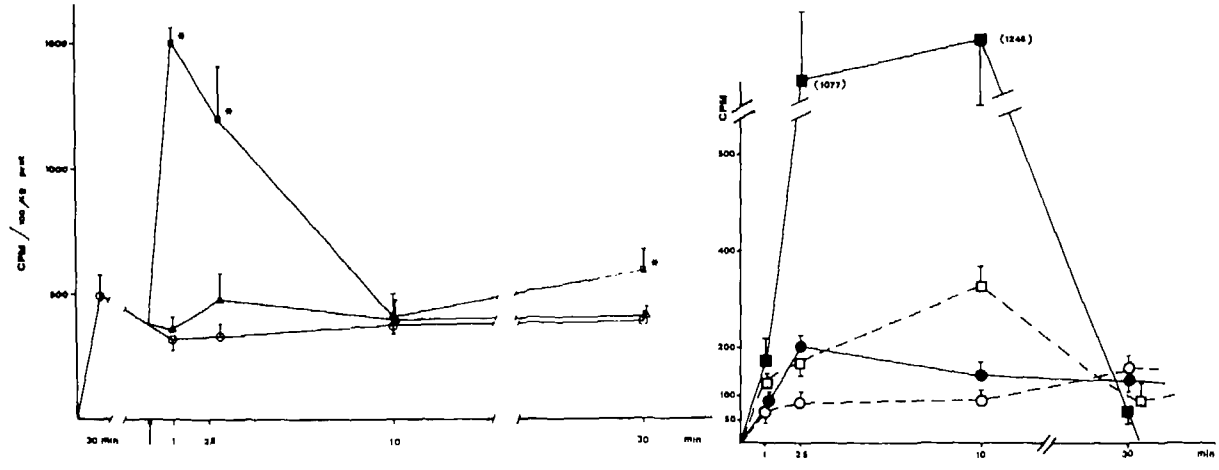

Figure 10. (*left*). $^{45}Ca^{++}$ uptake in normal bone cell cultures after stimulation with different doses of calcitonin. The hormone was added (*arrow*) after 30 minutes of preincubation (break on the left) with $^{45}Ca^{++}$ alone. Higher calcitonin concentrations (0.1; 1.0; 2.0 I.U./ml, ■—■), lower concentration (0.1 I.U.; ml, ▲—▲), and controls ($^{45}Ca^{++}$ without calcitonin, ○—○). The response of the bone-derived cells to hormonal stimulation is clearly dose-dependent. (Reproduced with permission.[58])

Figure 11. (*right*). $^{45}Ca^{++}$ uptake in normal bone cell cultures treated with sCT (0.5 I.U./ml) in the presence of an electromagnetic field (EF). Each point represents the mean ± SE of 5 assays performed in triplicate. Calcium incorporation induced by sCT treatment in bone-derived cells with (■—■) or without (●—●) EF; skin-derived fibroblasts with (□- - -□) or without (○- - -○) EF. (unpublished data.)

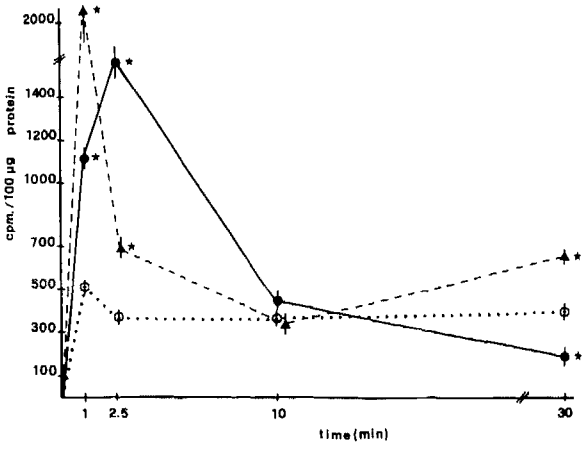

Figure 12. Effect of sCT (0.5 I.U./ml) (▲---▲) and db-cAMP (10^{-3} M) (●—●) on $^{45}Ca^{++}$ uptake in bone cell cultures. The lower line (O···O) shows controls incubated without either sCT or db-cAMP. Each point represents the mean ± SE (n = 8). Statistical evaluation compares these data with those obtained from cells incubated with $^{45}Ca^{++}$ alone ($p < 0.01$). The curve represents experiments performed on cells derived from a single biopsy. The results obtained from 4 other bone samples showed similar patterns of response, but different absolute values of radioactivity. (Reproduced with permission.[56])

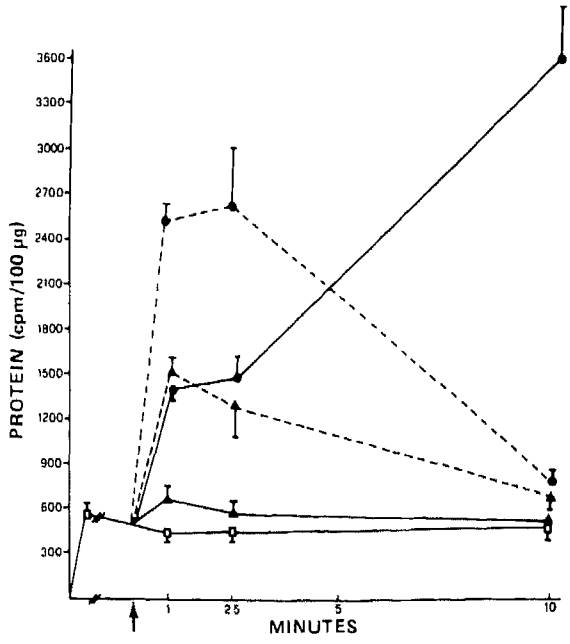

Figure 13. Effect of propranolol on calcium incorporation induced by sCT (0.6 I.U./ml) in normal and otosclerotic bone cell cultures: normal (triangles) and otosclerotic (circles) bone-derived cells after stimulation with calcitonin in the presence (solid lines) or absence (broken lines) of propranolol (2 µg/ml). Unstimulated controls are represented by □---□. (Reproduced with permission.[56])

treatment (Fig. 14). This suggested an alteration in the transducing mechanism between the stimulus, receptor, and cellular effector in cells derived from otosclerotic bone. On the basis of previous research,[62,66–68] that seems to demonstrate an adenylate cyclase (AC) dependence of calcitonin action, we undertook a detailed investigation of the functional activity of this type of cell membrane receptor.

Adenylate cyclase is an enzymatic complex that includes an extracellular receptor site, an intramembranous portion, and a catalytic unit on the cytoplasmic side (Fig. 15). When the hormonal molecules bind the specific receptor, they activate a catalytic unit capable of producing cAMP through a mediator sensitive to fluoride (F^-) and other intracellular molecules (G-proteins).[69] The last step of this reaction can be

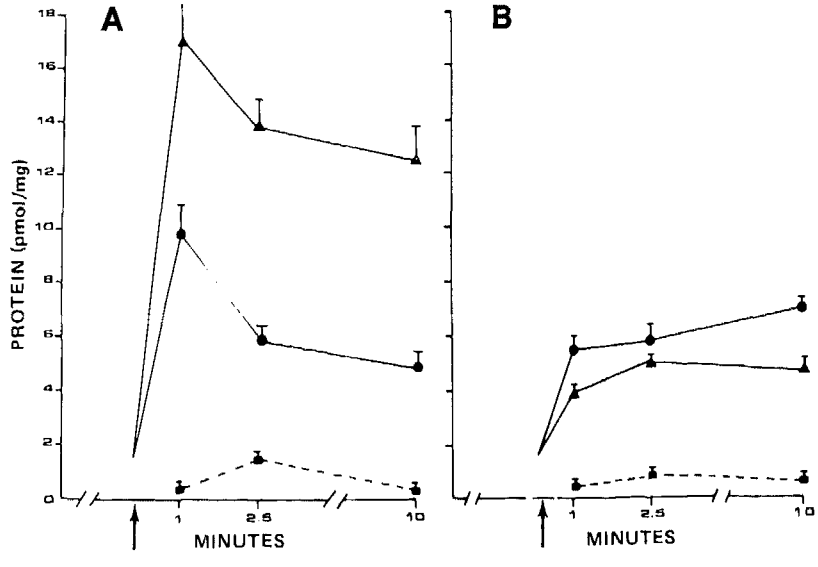

Figure 14. Cyclic adenosine monophosphate (cAMP) levels after hormonal stimulation (arrow) with calcitonin (0.5 I.U./ml, ▲—▲) and calcitonin + propranolol (0.5 I.U./ml and 1 µg/ml respectively, ●—●) compared to controls ($^{45}Ca^{++}$ without calcitonin, ■---■). A. Normal cell cultures B. Otosclerotic cell cultures. (Reproduced with permission.[56])

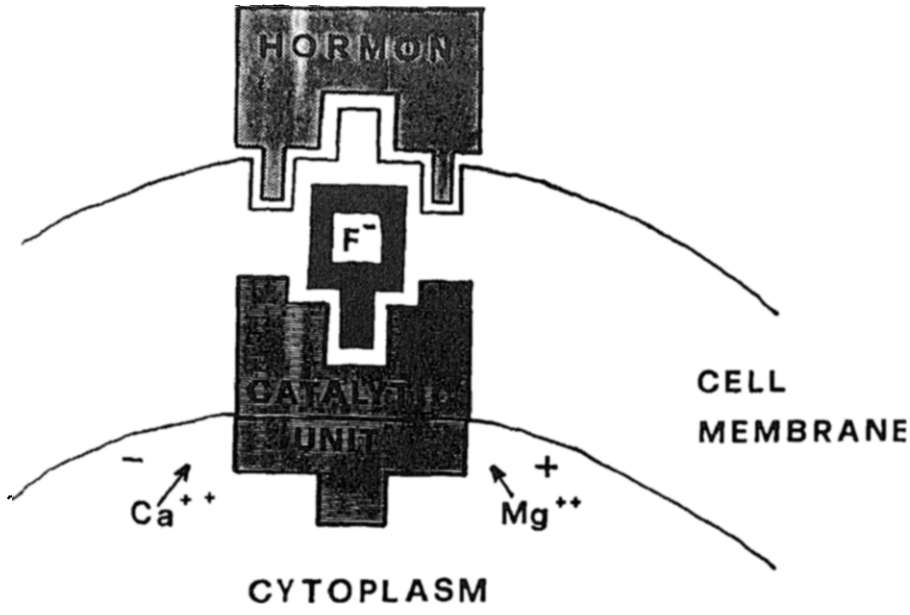

Figure 15. Scheme of the adenylate cyclase complex.

stimulated or inhibited by Mg^{++} or Ca^{++} respectively.[70,71] Therefore, the effect of F^-, Ca^{++}, and Mg^{++} on cAMP synthesis was evaluated in isolated membranes of normal otosclerotic bone-derived cells as well as in the skin fibroblasts used as controls. The results, summarized in Table 1, show that the increase of AC activity induced by Mg^{++} is much lower in otosclerotic cells than in normal bone-derived cells or fibroblasts. The Ca^{++} ion has almost the same inhibitory effect on all the three cell types, and F^- causes a strong AC activation in normal bone-derived cells, but a significant but clearly lower cAMP synthesis occurs in otosclerotic bone-derived cells. Adenylate cyclase activity is significantly depressed in cells derived from otosclerotic bone, and F^- ions can induce a partial recovery of enzymatic activity that, however, reaches less than 50% of the AC activity displayed by normal bone cells exposed to the same experimental conditions.

In conclusion, all experiments performed on the AC-dependent CT-receptor of otosclerotic bone-derived cells demonstrate an alteration of this enzymatic complex. Correspondingly, this complex may play a crucial role in the pathogenesis of otosclerosis. We intend to continue our investigations in this direction.

TABLE 1. Specific Activity of Adenylate Cyclase in Bone Cells

Cofactor	Bone		
	Otosclerotic	Normal	Fibroblasts
Mg^{++} (5.0 mM)	2.55 ± 0.4	6.60 ± 0.9	10.18 ± 1.5
Ca^{++} (5.0 mM)	0.69 ± 0.1	0.63 ± 0.2	0.96 ± 0.2
NaF (5 mM)	6.92 ± 1.0	19.00 ± 2.9	8.40 ± 1.3

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