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A three-dimensional ultrastructural study of osteoid-osteocytes in the tibia of chick embryos

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Summary. Morphology and ultrastructure of osteoid-osteocytes were studied in serial thin sections (700–800 Å thick) of periosteal woven bone in tibiae of 15-day-old chick embryos. The three-dimensional shapes of 21 partially, and of one fully sectioned cell were reconstructed manually and by means of a computer-assisted image analyser.

Osteoid-osteocytes are active cells engaged in organic matrix secretion and calcification. Like osteoblasts, their activity seems to be polarized towards the mineralization front, as shown by the presence of cytoplasmic processes on their mineral-facing side and by the position of the nucleus toward the vascular side of the cytoplasm. Cellular processes directed towards blood vessels appear only at a later stage, i.e. when the mineralization starts to spread all round the cell.

The asynchrony in formation, together with the observed differences in morphology suggest the hypothesis that the cellular processes of the mineral-facing side are mainly involved in bone formation and those of the vascular side in cell nutrition.

Key words: Osteocyte differentiation – Osteoid-osteocyte – Three-dimensional reconstruction – Transmission electron microscopy – Chick embryo

It has been known since the last century (Gegenbaur 1864; Waldeyer 1865a,b) that the osteocyte originates from the osteoblast. However, only in more recent years has the process of osteocyte differentiation been widely investigated by means of different techniques, from both the morphological and the functional point of view. Studies with both the light and electron microscope have shown that the dendritic shape of the mature osteocyte is progressively derived from the original rounded osteoblast through conspicuous morphological and ultrastructural changes. The cellular body reduces in size in parallel with the formation of the cytoplasmic processes; the amount of the cytoplasmic organelles also decreases, whereas the nucleus-to-cytoplasm ratio increases (Dudley and Spiro 1961; Hancox and Boothroyd 1965; Cooper et al. 1966; Cameron 1972; Rasmussen and Bordier 1974; Nijweide et al. 1981).

Morphometric investigations carried out in our laboratory have shown that any osteoblast of an osteogenetic layer may enter the bone matrix to become an osteocyte and that, independently of its initial dimension, the cellular body reduces by about 30% at the stage of osteoid-osteocyte and by about 70% when the osteocyte reaches full maturity. Thus the size of the osteocytes appears to be proportional to the size of the original osteoblasts (Marotti 1976). Studies on the periosteal bone of young rabbits using H_3-thymidine and H_3-glycine have shown that it takes approximately three days for an osteoblast to become an osteocyte and that, during this period, it manufactures three times its own volume of matrix (Owen 1963).

Although the process of osteocyte differentiation now appears to be better understood many aspects are still obscure. We do not know, for instance, what kind of stimulus induces a given osteoblast to become an osteocyte or from where this stimulus comes. Also the pattern of formation of the cytoplasmic processes is quite unknown, whether or not: (a) they start to radiate simultaneously from the whole periphery of the cell; (b) they stop growing before the mineralization of surrounding organic matrix is completed; (c) their arborization is symmetrical on both mineral-facing and vascular sides of the cell; (d) the amount of cytoplasm they contain corresponds to the decrease in volume of the cellular body.

To collect information that may throw light on these problems, we have recently started a series of three-dimensional investigations on the morphology of osteocytes at different stages of differentiation, using transmission electron microscopy. In the present paper data concerning osteoid-osteocytes are reported.

Materials and methods

Ultrastructural analyses were performed on the periosteal woven bone at the mid-diaphyseal level of the tibiae in 15-day-old chick embryos. Cross-sections (2 mm thick) of the diaphyses were fixed with 4% paraformaldehyde in 0.13 M phosphate buffer for 2–3 hours, postfixed in 1% osmium tetroxide in 0.13 M phosphate buffer for about 2 hours, and embedded in epoxy resin (Durcupan ACM). All the specimens were serially sectioned with a diamond knife mounted in an Ultracut-Reichert microtome. To prevent demineralization of the bone, the water bath on which the sections (700–800 Å thick) were floated was kept above pH 7. Serial sections were mounted on Formvar-coated, carbon-coated copper grids with a central hole of 1.5 mm diameter. The sections were then stained with 1% uranyl

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acetate and lead citrate, and examined and recorded by use of a ZEISS EM 9 electron microscope. Micrographs were printed at a total magnification of ×9500.

Not less than 75–80 sections are needed to cut serially through an entire osteoid-osteocyte. The observations reported in this paper refer to 21 cells partially sectioned at different levels and to 1 cell fully cut in 87 sections. The three-dimensional reconstruction of the partially and completely sectioned osteoid-osteocytes were made: (a) by superimposing on transparent sheets the outlines of the cell membrane and of the nuclear membrane manually recorded from all the photographs of each series of sections; (b) by means of an Image Analyser TESAK VDC 501 and PDP 11/23 Digital Computer. The program superimposed the stored outlines (1 every 3) of a given cell, then deleted outline points masked by preceding sections in order to see only edges which were not obscured by higher sections. By tilting the apparent angle at which the sections are viewed, the computer rotated the image.

Results

Osteoid-osteocytes appear to be surrounded on all surfaces by tightly packed unmineralized collagen fibers, the diameters of which increase from the osteoblastic lamina (170–260 Å) towards the mineralization front (500–700 Å). The osteoid-osteocytes selected for the three-dimensional reconstruction were close to the mineralization front and partially surrounded by patches of calcification on their lateral sides; a thin layer of unmineralized matrix separated the cell membrane from the mineral salts. Small clusters of crystals were occasionally present also on their vascular surface facing the osteoblasts (Fig. 1). Like the adjacent osteoblasts, they contain an abundance of granular endoplasmic reticulum, numerous free ribosomes, many mitochondria and a well developed juxtanuclear Golgi apparatus (Fig. 2). The nucleus is usually located towards the vascular side and shows a peripheral concentration of chromatin and one or two prominent nucleoli (Figs. 1, 3).

The cytoplasmic processes are numerous, with few branches, and originate almost exclusively from the mineral-facing side of the osteoid-osteocytes; most of them extend for a short distance into the mineralization front, the others radiate parallel to it. The many small pseudopodia-like protrusions arising from the osteoblast plasma membrane (Fig. 4) are not present in osteoid-osteocytes at the stage studied in this paper, and their cellular processes are longer than those of the osteoblasts and not covered by globular structures (matrix vesicles). Cytoplasmic processes were occasionally observed on the vascular side but even when they appeared on the vascular side they were always present in a much greater number on the mineral-facing side. They become a constant feature of the vascular side of the osteocyte only at a later stage, when the mineralization spreads all around the cell. The cellular processes arising from the vascular side seem to be longer and more slender than those of the mineral-facing side (Fig. 5).

This asymmetry of the distribution of cytoplasmic processes was observed in the 21 partially sectioned and the fully sectioned osteoid-osteocytes (Fig. 3).

No significant difference was found between the three-dimensional reconstructions of the shape of the cells made manually and those made by the image analyser (Fig. 6). The latter method facilitated the measurement of morphometric data for the fully sectioned osteoid-osteocyte: *cell protoplasm* 237 μm^3; *cytoplasm* 187 μm^3; *nucleus* 50 μm^3; *cytoplasmic processes* 24 μm^3; *cellular body* 213 μm^3; *nucleus-to-cytoplasm ratio* 0.27; *cell surface* 209 μm^2; *cytoplasmic process surface* 64 μm^2; *diameters:* x 10 μm, y 8 μm, z 5 μm (the x and z diameters are respectively parallel and perpendicular to the mineralization front).

Discussion

The ultrastructure of osteoid-osteocytes has already been described in literature (Dudley and Spiro 1961; Nijweide et al. 1981). It is generally admitted that they are active cells still engaged in secretion of organic matrix. In fact, they closely resemble osteoblasts with respect to the cytoplasm organelle content. It should be mentioned in this connection that during differentiation of osteoblasts to osteocytes there are many intermediate forms characterized by a gradual decrease in the amount of cytoplasmic organelle. The osteoid-osteocytes selected for three-dimensional analysis in this investigation were certainly not at the earliest stage of differentiation, because they were already close to the mineralization front and also partially surrounded by mineral salts on their lateral sides. Although these cells have reached a certain degree of maturation, their morphology does not suggest a diminution in secretory activity compared with osteoblasts. A decrease in organelle amount is clearly apparent first in pre-osteocytes almost completely surrounded by minerals.

The major finding of the present three-dimensional study concerns the asynchrony in the formation of the cytoplasmic processes between the mineral-facing and vascular sides of osteoid-osteocytes. This is shown by the fact that cellular processes on the vascular side only appear after they are already present on the mineral-facing side. Cellular processes radiating only from the mineral-facing side of osteoblasts have been described by many authors (Dudley and Spiro 1961; Cameron 1972; Cooper et al. 1966; Ornoy et al. 1980; Zylberber and Castanet 1985). The osteoblasts we observed in chick bone also show an irregular outline due to many cytoplasmic protrusions interdigitating with those from adjacent cells. However, even the longest of these protrusions radiating towards the osteoid seam, differ from the protrusions arising from osteoid-osteocytes. Most osteoblastic protrusions appear to be pseudopodia, probably involved in the extrusion of calcifying matrix vesicles, as is shown by the fact that they are surrounded by many globular structures most of them containing crystals (Fig. 4). Matrix vesicles as the locus of initial calcification in avian bone have already been reported in literature (Ascenzi et al. 1963; Ascenzi 1964; Decker 1966; Bonucci 1971).

On the basis of the arrangement of the three main cell compartments (nucleus, endoplasmic reticulum, Golgi apparatus) it has been rightly maintained by many authors (Dudley and Spiro 1961; Cameron 1972; Pritchard 1972; Jones 1974; etc.) that the activity of osteoblasts is polarized in relation to the calcifying matrix. The demonstration in this paper that osteoid-osteocytes, even those adjacent to the mineralization front, have the nucleus located close to the vascular side and the cellular processes radiating from the opposite side only, strongly indicates that their activity is still polarized towards the calcifying bone matrix, at least

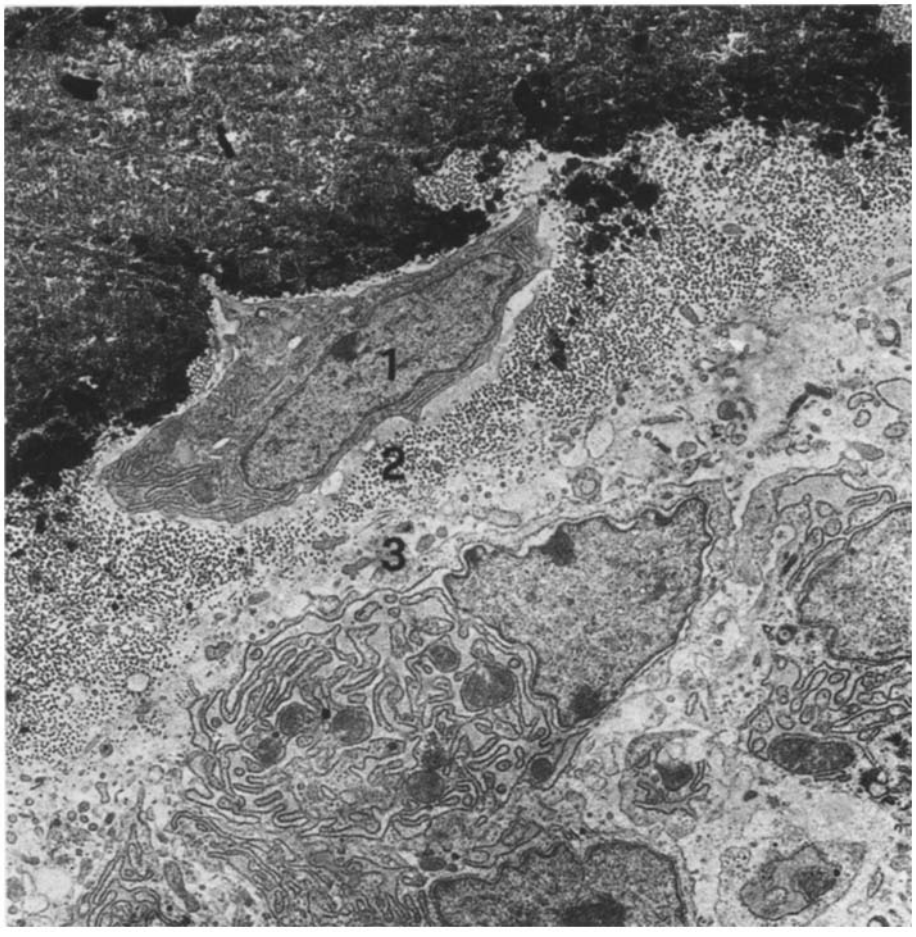

Fig. 1. Osteogenic surface in periosteal woven bone of the tibia from a 15-day-old chick embryo showing the type of osteoid-osteocyte selected for three-dimensional reconstruction. From top to bottom note: the osteoid-osteocyte showing a regular outline (*1*), the osteoid seam made up of an outer layer of cross-sectioned collagen fibers (*2*), and an inner layer containing many short cellular projections and matrix vesicles originating from the osteoblast (*3*). Note also that the diameter of the collagen fibers increases towards the mineralization front. × 7700

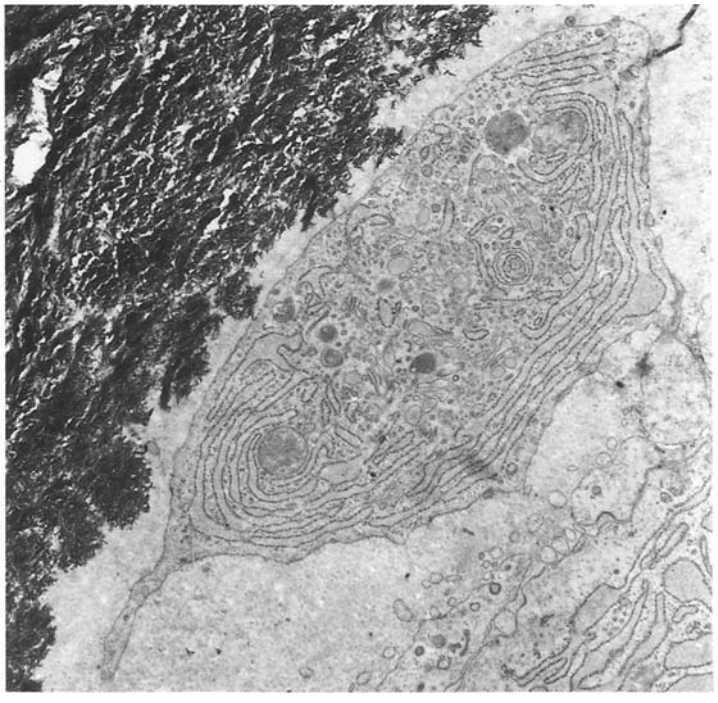

Fig. 2. Fine structure of an osteoid-osteocyte. The cellular body contains a well developed Golgi apparatus surrounded by many cisternae of granular endoplasmic reticulum. × 9500

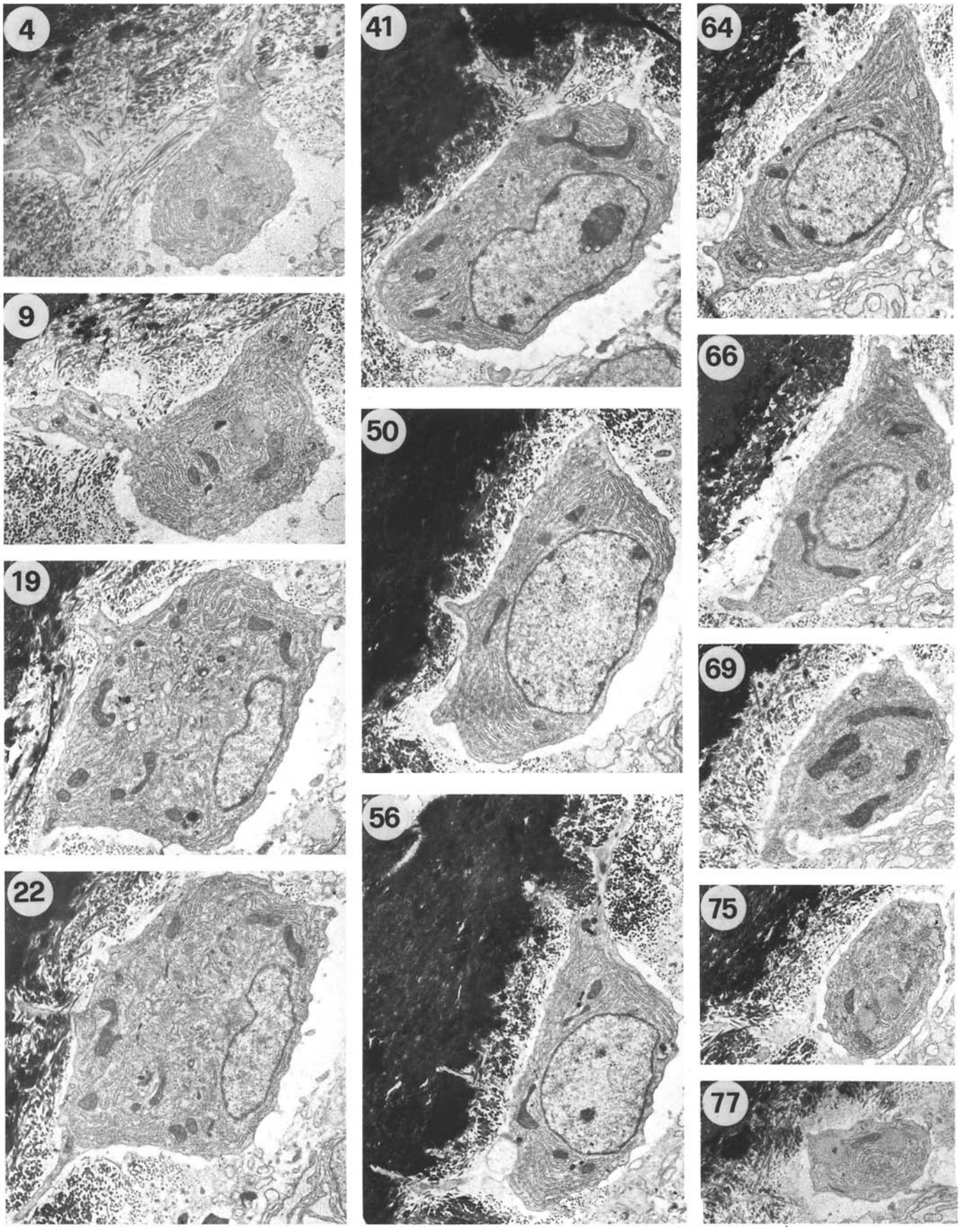

Fig. 3. Twelve sections selected from the series of 87 consecutive sections through an osteoid-osteocyte. The number in each photograph indicates the level of the corresponding section in the whole series. Note the presence of cytoplasmic processes radiating only towards the mineralization front, the eccentric position of the nucleus and the abundance of cytoplasmic organelles. The three-dimensional shape of this osteoid-osteocyte is shown in Fig. 6. × 5300

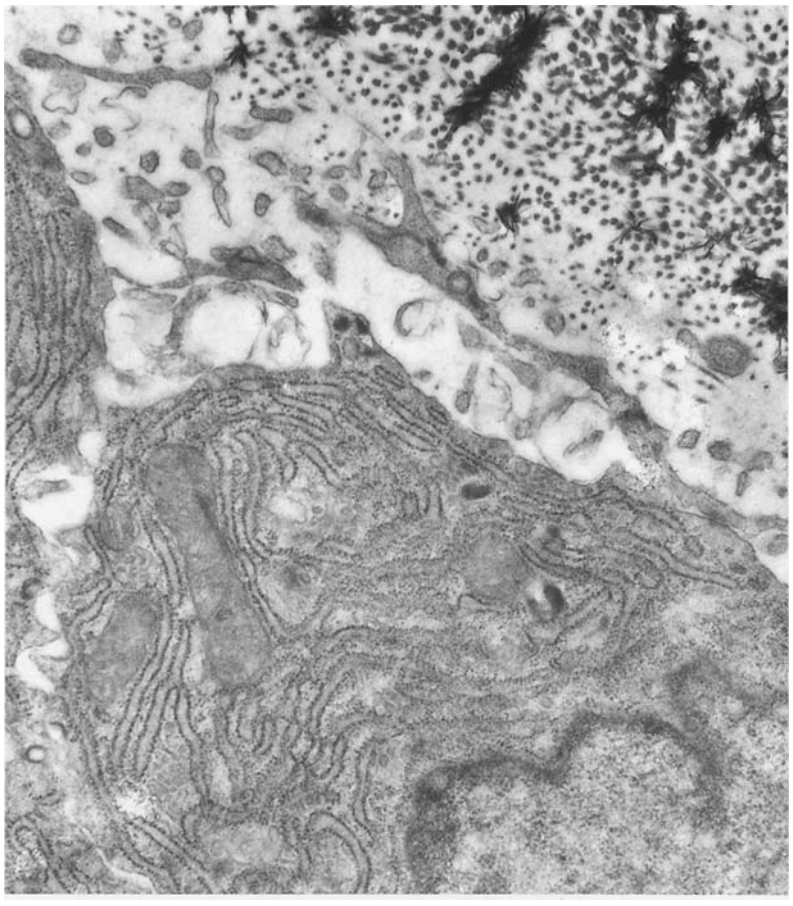

Fig. 4. Part of an osteoblast facing the osteoid seam. Many pseudopodia-like protrusions and globular structures (matrix vesicles) are present on the side of the cell facing the osteoid seam. Among the collagen fibers some matrix vesicles are filled with crystals. Note also, on the left side of the cell, very short protrusions of the plasma membrane interdigitating with those of the adjacent osteoblast. × 18000

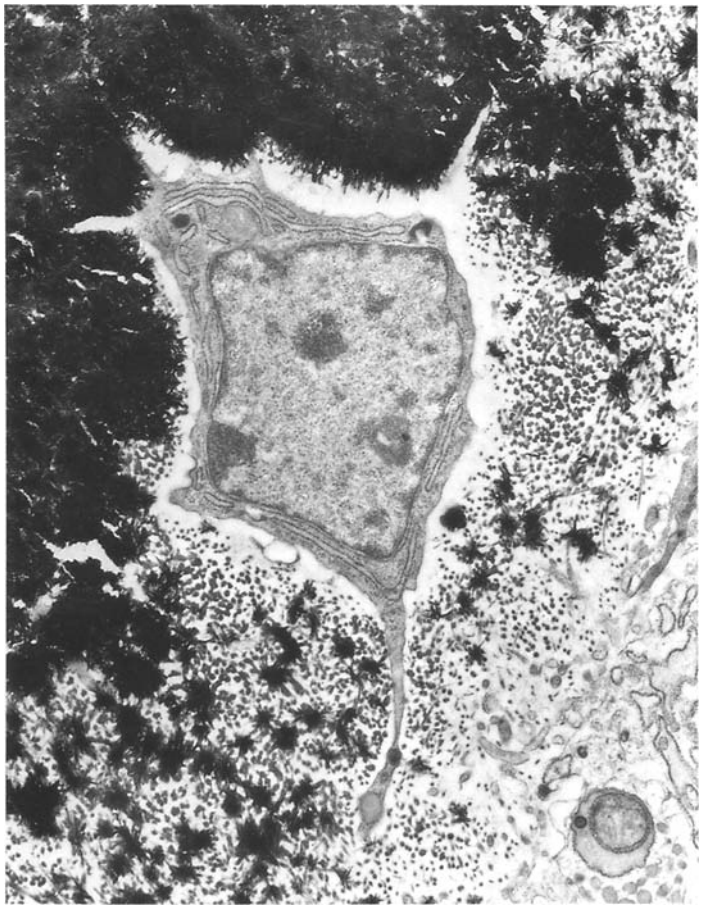

Fig. 5. Pre-osteocyte almost completely surrounded by clusters of crystals; this cells is at a more advanced stage of differentiation compared with the osteoid-osteocytes three-dimensionally studied in this investigation. Note the long and slender cytoplasmic process arising from the cell vascular side and the regular outline of its plasma membrane. × 9500

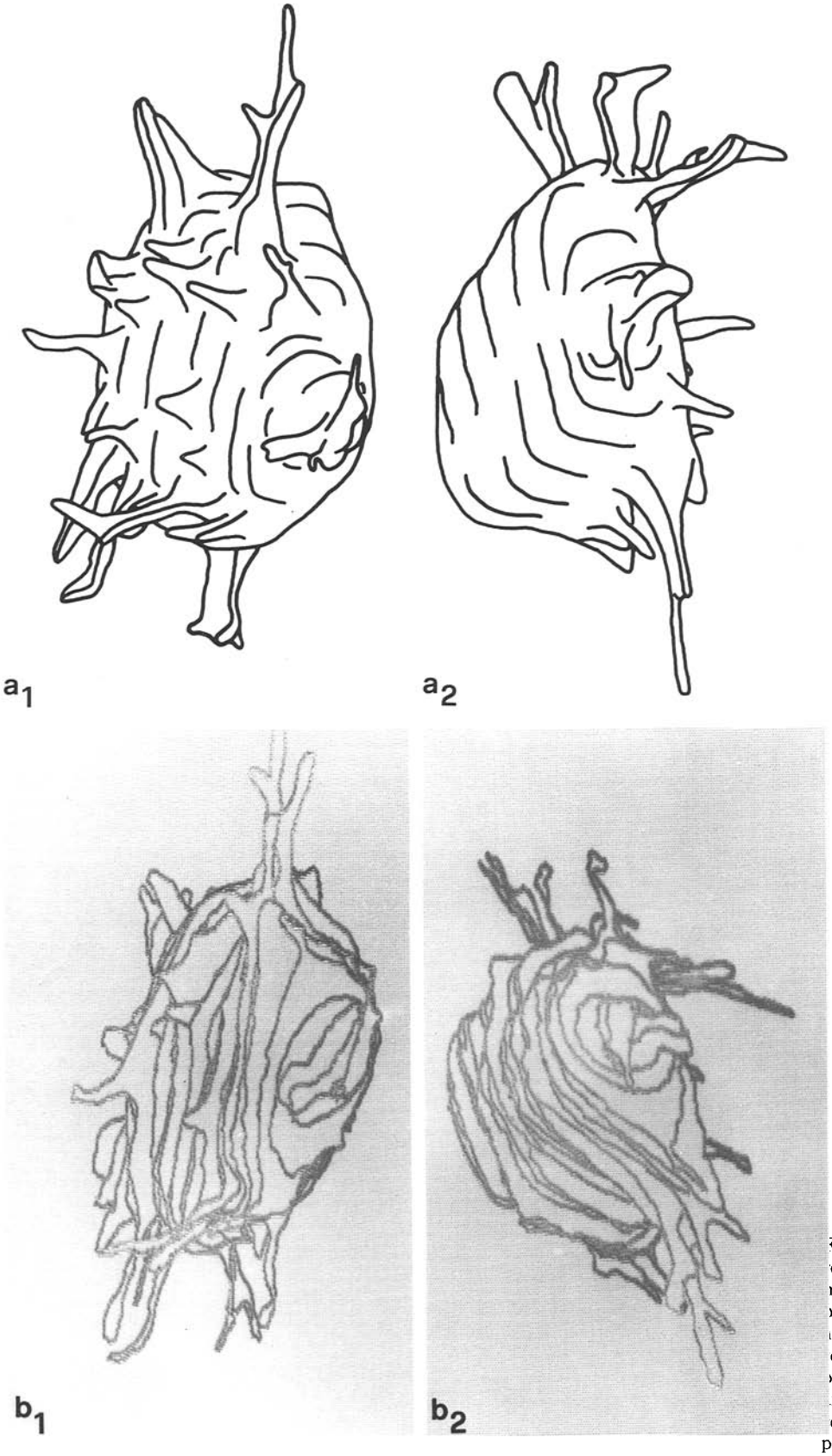

Fig. 6a, b. Three-dimensional reconstruction of an osteoid-osteocyte made (**a**) manually and (**b**) by means of the computer-assisted image analyser. In $\mathbf{a_2}$ and $\mathbf{b_2}$ the cell is rotated with respect to $\mathbf{a_1}$ $\mathbf{b_1}$ by 180° on the *y* axis and by 90° on the *z* axis. The mineral-facing side of the cell is the one having cytoplasmic processes

until they start to be surrounded by mineral salts on their vascular side as well.

Dudley and Spiro (1961) suggested that the cytoplasmic processes of osteoid-osteocytes, like those of the osteoblasts, may affect the polymerization of proteoglycans. On the other hand, Bordier and co-workers (1976) and Nijweide and co-workers (1981) assign to osteoid-osteocytes an important role in the initiation and control of matrix calcification. We agree that osteoid-osteocytes may participate in matrix secretion and mineralization, and may also be involved in the orientation of collagen fibers, as suggested for the osteoblasts by Jones and co-workers (1975, 1976, 1977). We believe, however, that they perform all these functions mainly from their mineral-facing side. In fact, for both osteoid-osteocytes and pre-osteocytes at a later stage of maturation (see Figs. 1, 2, 5) the plasma membrane facing the vessels shows neither pseudopodia-like protrusions nor calcifying matrix vesicles. Thus the formation and mineralization of the osteoid matrix located between osteoid-osteocytes and osteoblasts would seem mainly to depend on the activity of the latter.

The osteoid-osteocyte in woven bone of chick embryo is therefore an asymmetric cell as regards shape and activity. The short cellular processes that the cell radiates from the mineral-facing side seem to be involved in bone formation, while the long processes which later radiate towards the blood vessels probably have a nutritional function. This suggestion is consonant with the finding that the number of canaliculi arising from osteocyte lacunae is greater from the vascular walls than from the opposite ones facing the cement line (Remaggi et al. 1981; Remaggi and Zaffe 1982; Marotti et al. 1985). Further three-dimensional studies of the entire series from pre-osteocytes to mature osteocytes are needed not only in avian woven bone but also in lamellar bone from mammals to elucidate the process whereby osteoblasts differentiate to osteocytes, and to detect whether an asymmetrical shape persists in mature osteocytes with respect to both number and length of the cytoplasmic processes.

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