Ectopic Mineralization in Fibroblast Cultures*

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Received June 20, 1983

Summary. A human gingival fibroblast cell line was cultured in a DM-153 medium supplemented with 10% bovine serum. The cells in culture showed both intracellular and extracellular ectopic mineralization. Ultrastructurally, mineralization began within the round and irregularly shaped vesicles contained in large cytoplasmic vacuoles of fibroblasts. The first identifiable needle-shaped crystal was deposited on a point of the inner leaflet of the vesicle membrane. With the increase in size of the crystals, the membranous envelopes were gradually lost, and lattice planes (8.2 Å) occurred within the crystal thickness. Crystals radiating from each mineralization center formed mineralized spherules in cytoplasmic vacuoles.

The crystal clusters were extruded from the cells through membrane fusion or cellular degeneration in long-term cultures. These liberated clusters formed extracellular mineralizing matrices around the fibroblasts. In extracellular mineral deposition, the initial crystals arose within extracellular matrix vesicles, but were not associated with collagen, elastic fibers, and any other structures.

These findings indicate that mineral accumulation and phase transformation of amorphous mineral to a crystalline form take place within the vesicular structures. This provides additional evidence for the view that the initiation of calcification and mineralization requires a microenvironment limited by a membranous (vesicular) structure derived from cells.

In 1967, ANDERSON and Bonucci independently demonstrated that longitudinal septa of growth cartilage contain extracellular membranous matrix vesicles, which play a role in the initial crystallization of apatite mineral. Since then, there have been many reports supporting this concept. The matrix vesicles are now known to have an essential role in the primary calcification of normal cartilage, bone, dentin, cementum, and other calcifying tissues in a large number of animal species as well as in man (see Anderson, 1976a, b, for reviews).

Matrix vesicles or analogous structures also have been found in a variety of pathological ectopic mineralizing tissues such as the aorta (Kim, 1976), osteosarcoma (Lee, 1974), and chondrosarcoma (SchaJowicz et al., 1974), and in bone- or cartilage-derived cell cultures (Binderman et al., 1974; Williams et al., 1980; Suzuki et al., 1981; Nijweide et al., 1982; Sudo et al., 1983). However, it has not been previously shown clearly whether the first identifiable microcrystals of apatite mineral in ectopic miner-

*This work was supported in part by a grant from the Japanese Ministry of Education (No. 58480368).
alization might deposit 1) within the matrix vesicles, 2) in the vicinity or in close apposition to the surface of the vesicles, or 3) on fibrillar or other structures in the matrix. Moreover, the role of the matrix vesicles in ectopic mineralized tissues has not been clearly established.

Williams et al. (1980) suggested that fibroblasts and other cells cultured under certain conditions might provide a useful model of ectopic mineralization in vitro. Recently we identified a fibroblast cell line which deposits minerals under normal culture conditions. We have now examined the ultrastructural relationship between the initial mineral deposition and vesicular structures, and the chemical nature of the mineral constituents has been analyzed by electron microscopy and electron probe x-ray microanalysis.

MATERIALS AND METHODS

Cell culture
Cells used in this study were human gingival fibroblasts, HGF-7 (2), derived from the non-inflamed gingival tissue of a young female. The cells were cultured on 35 mm Lux plastic culture dishes in a DM-153 medium (Kyokuto Pharmaceutical Ind. Co., Tokyo, Japan) supplemented with 10% bovine serum (Flow Laboratories, Inc., Virginia, USA) and penicillin G (100 units/ml). They were incubated in a humidified 5% CO₂-95% air mixture at 37°C. The medium was replaced every three days.

Electron microscopy
Cells were fixed in situ on plastic dishes for 2 hrs with 2% glutaraldehyde-2% paraformaldehyde in calcium chloride-containing 0.1 M cacodylate buffer (pH 7.3) at 4°C after 1, 5, 10, 15, 20, 30 and 40 days of culture and then rinsed with 7% sucrose in the same buffer (0.05 M). The cultures were postfixed in 1% osmium tetroxide in the same buffer at 4°C for 2 hrs and then stained en bloc in 0.5% uranyl acetate for 1 hr at 25°C. In some cultures this procedure was omitted. They were then dehydrated through a graded ethanol series and embedded in Epon 812 as previously described (Yajima and Rose, 1977). The polymerized blocks were cut with a Porter-Blum MT-II ultramicrotome and stained with uranyl acetate and lead citrate. The thin sections were examined and photographed in a JEOL 100-CX electron microscope.

Energy dispersive x-ray analysis
Selected, unstained grids were carbon-coated on the under-surface of the sections to stabilize them for electron probe microanalysis. Microanalysis was performed with a JEOL 100-CX electron microscope fitted with an Ortek energy-dispersive x-ray spectrometer. The microprobe conditions were: 100 KeV accelerating voltage, 15 nm spot size, and 100 s analysis time. The portions to be analysed were selected by observing scanning transmission images.

RESULTS

Under normal culture conditions, HGF-7(2) human gingival fibroblasts reached confluence in 6–7 days. The cells proliferated in post-confluent cultures and formed multiple layers. Small mineral depositions in cytoplasmic vacuoles were observed in
cultures at 3–5 days. These intracellular deposits increased in number and size with incubation time, then followed by some extracellular mineral deposits which could be found in long-term cultures. This HGF-7(2) cell line was characterized by extensive intracellular and extracellular mineralization.

In earlier stages of mineralization, several small round, ovoid, flat or irregular-shaped vesicles or saccules were observed within the large cytoplasmic vacuoles of the fibroblasts (Fig. 1). These membrane-bound intracellular structures had extremely variable diameters ranging from 30 to 200 nm. The membrane sac comprised a trilaminar structure with a typical clear band sandwiched between two dense bands (outer- and inner-leaflets), being identical in appearance to the vacuole sac and plasma membrane of fibroblasts. The interior of these vesicles varied considerably in appearance, ranging from partially empty to containing amorphous materials of variable electron density.

At this stage crystals were not present within the vesicles, although amorphous electron-dense deposits were seen associated with the inner leaflet of the vesicle membrane. Careful examination showed that fine, thread-like structures first occurred on a certain point of the inner leaflet of some intracellular vesicles (Fig. 2). The membrane limiting the vesicles was analyzed on electron microscopic films with a microdensitometer (Fig. 3). The widths of the three layers measured, from the half-height of the bands, 2.5, 3.0, and 2.0 nm from the outside to the inside of the vesicle membrane.
Fig. 2. Intravacuolar vesicles in the area of initial mineralization. A fine, thread-like deposit is seen on the inner leaflet of a vesicle (arrowhead). The membrane structure of the vesicles is also shown in Figure 4. × 162,000

Fig. 3. Legend on the opposite page.
(a total width of 7.5 nm), coincided with the corresponding parts of the vacuole sac and plasma membrane. In contrast, the vesicles containing electron-dense materials associated with their inner leaflet showed a highly asymmetric trilaminar membrane: the thickness of the inner electron-dense layer increased to 4.5 nm. In the saccules enclosing crystal-like structures, the central electron-dense band measured 2.5 nm in thickness, but the inner leaflet could not be distinguished from the dense band.

The membranous envelopes became elongated along with the increasing length of crystal-like structures (Fig. 2, 4, 5). The inner leaflet of the envelopes, therefore, came into close contact with the crystal-like structures, whereas the outer leaflet appeared like a sheath or coat around the structures. At this stage, the crystalline structures were clearly visible in unstained sections. The deposits were likely mineral in nature (Fig. 5). There was a considerable variation in the shape of the saccules, this seeming to depend on the location of the enclosed crystalline structure. However, only one crystalline structure was deposited within one vesicle in these mineralizing fibroblast.

Fig. 4. A. Needle-shaped crystalline structures developed within the membranous envelopes. The vesicles further contain varying amounts of amorphous materials. ×69,000. B. Higher magnification of the membranous envelopes bearing crystalline structures shown in A. The outer leaflet appears like a sheath (arrows) surrounding the crystals. ×190,000

Fig. 3. Membranous structures of discrete vesicles analyzed on the electron microscopic film with a microdensitometer (see text for details). The curves a, b and c correspond to the membranous structures with the same labels in Figure 2; the arrows in Figure 2 indicate the analyzing direction. a. A vesicle showing the trilaminar membrane structure. b. A vesicle including an electron-dense material associated with its inner leaflet. The trilaminar appearance of the membrane is highly asymmetric. c. A saccule including a crystal-like structure. Note the band of conspicuous electron density; no inner leaflet structure is recognized.
It also was evident that initial crystallization was confined to the intracellular vesicles and no crystalline structure occurred associated with intracellular fibrous elements or other structures.

With the increase in the size of the crystals, their envelopes are gradually lost. The crystals had an average thickness of 5 nm, and their lengths varied greatly, ranging between 50 and 160 nm. Two or more lattice planes (8.2 Å periodicity) were observed within the crystals under high magnification (Fig. 6).

At the next stage, the lattice planes increased in number within the crystals, and the sheath structure completely disappeared from their surfaces. The needle-shaped crystals were transformed to hexagonal ones (Fig. 7). The average thickness and width of the mature crystals were 7 to 10 nm and 15 to 70 nm, respectively. Microdensitometry indicated that the average spacing of the 100 lattice planes of these crystals was 8.2 Å, coinciding with that of hydroxyapatite crystals.

As intracellular mineral deposition continued, discrete mineralized spherules coalesced to form more complex clusters in the cytoplasmic vacuoles (Fig. 8). The entire vacuoles then gradually became filled with crystals. Many membrane-bound intracellular bodies containing varying amounts of crystals were observed in cultures at 10–40 days. Subsequently, these intracellular clusters of crystals were extruded from the cell through membrane fusion or cellular degeneration. These clusters became
the extracellular mineralization foci (Fig. 9). Extracellular matrix vesicles were commonly seen in association with crystal aggregates and also appeared to be a site for initial crystal deposition.

The elemental components of the intracellular and extracellular mineral deposits were analysed by energy-dispersive x-ray microanalysis (Fig. 10). The control non-mineralized matrix contained osmium (1.91 KeV) and traces of sulfur (2.31 KeV) and chloride (2.62 KeV), which had been introduced during sample preparation. The mineral deposits appeared to be composed primarily of calcium (Kα, 3.69 KeV; Kβ, 4.00 KeV) and phosphorus (2.01 KeV). Furthermore, these crystals were identified by their electron diffraction patterns as hydroxyapatite or octacalcium phosphate.

**DISCUSSION**

The present study demonstrates that the electron-dense deposits in cultured gingival fibroblasts are associated with the membranes of vesicles and that initial mineralization proceeds in these intracellular structures. Similar deposits have been described associated with the plasma membrane of osteoblasts and matrix vesicles in alveolar bone (Dougherty, 1979), with the plasma membrane of pre- and young-odontoblasts and dentinal matrix vesicles (AlMuddaris and Dougherty, 1978), and with the plasma...
membrane of various cells involved in calcium metabolism (Oschman and Wall, 1972; Hillman and Llinas, 1974; Oschman et al., 1974; Plattner, 1975; Boquist, 1977).

Our results suggest that amorphous deposits of high electron density accumulate at calcium-binding sites which are present in the membrane-bound structures. Although the nature of these calcium-binding sites within the membrane is unknown, several possibilities have been suggested, principally in relation to phospholipids, phosphoproteins, calcium-binding proteins, and the trapping action of phosphate (Almuddaris and Dougherty, 1979). Firstly, biochemical analysis of isolated epi-
physeal cartilage matrix vesicles has documented the presence of several phospholipids, such as phosphatidyl serine, which have a high affinity for calcium (Perss et al., 1974; Wuthier, 1975, 1976). In vitro studies have further suggested that the vesicle lipids might be involved in the initial binding and phase separation of calcium phosphate, as well as its conversion to crystalline apatite (Wuthier, 1976). Vogel and Boyan-Salyers (1976) indicated that acidic phospholipids such as phosphatidyl serine are constituents of the inner leaflet of the membrane. It seems not accidental that the amorphous mineral deposits were associated with the inner leaflet of the membrane limiting the vesicles (Fig. 2, 3).

Secondly, phosphoproteins which have affinity for calcium have been reported to be present in dentin (Veis and Perry, 1967; Butler et al., 1972; Weinstock and Leblond, 1973) and enamel (Seyer and Glimcher, 1971).

Thirdly, recent studies have demonstrated proteins containing the calcium-
phospholipid-binding amino acid, \( \gamma \)-carboxyglutamic acid, in bone and numerous other sites of pathological and ectopic mineralization (Lian et al., 1976, 1977; Hauschka and Gallop, 1977). Since \( \gamma \)-carboxyglutamic acid-containing protein is present in various sites of normally calcified and ectopically mineralized tissues and absent in unmineralized ones, the presence of this amino acid may cause the primary nidus for mineral deposition.

Finally, it has been suggested that calcium is trapped by phosphate liberated at

Fig. 8. An intracellular mineral deposit seen in a cytoplasmic vacuole of a fibroblast after 10-days of culture. This mineralized structure is apparently formed by fusion of crystal clusters. \( \times 38,000 \)

Fig. 9. Three clusters of crystals liberated from the cell after 30-days of culture. Note the close association of the extracellular crystals clusters with the plasma membrane. \( \times 21,000 \)
the site of membrane phosphatase activity (Robinson, 1923; Anderson and Reynolds, 1973). These enzymes are believed to increase the local phosphate concentration, allowing precipitation of calcium phosphate first in an amorphous phase, and later in a crystalline hydroxyapatite phase (Irving, 1976). Cytochemical studies have confirmed the presence of alkaline phosphatase and ATPase activity in matrix vesicles (Matsuzawa and Anderson, 1971; Larsson, 1973).

It thus seems possible that a gradual, yet active accumulation of calcium and phos-

Fig. 10. A. Scanning transmission mode electron micrograph of an intracellular mineralized area in a fibroblast. Unstained. x 11,000. B and C represent x-ray energy spectra obtained from the intracellular mineralized area (B) and an adjacent region of the cytoplasm (C) (corresponding to the sites indicated by arrows b and c in A. B. Note the occurrence of large peaks for calcium (CA), phosphorus (P), and copper (CU), which is derived from the specimen grid. C. Note traces of sulfur (S) and chloride (CL) from the embedding materials and osmium (OS) from the secondary fixative.
phate ions in the membrane-bound structures would lead to the formation of the electron-dense deposits associated with the inner leaflet and finally to precipitation of apatite.

We know little about the origin or biogenesis of these intracellular membrane-bound structures. Some of the vesicles appeared to arise as a result of budding from the cytoplasmic vacuoles or sacs of fibroblasts. Others may be the degenerative products of the plasma membrane of cells and their organelles. These membrane-bound structures closely resemble the extracellular matrix vesicles described in the calcification process in a variety of normal tissues such as epiphyseal cartilage, bone and dentin.

SUZUKI et al. (1981) reported that an increased phosphate ion concentration in the culture medium of a growth cartilage cell system induced apatite crystals associated with collagen fibrils, whereas coculture of the cartilage cells with bone marrow cells produced crystals associated with matrix vesicles. Thus, the former type of mineralization is regarded as an artificial phenomenon induced by a physicochemical process. GLIMCHER and KRANE (1968) provided evidence that collagen was a template for the heterogeneous nucleation of bone mineral, and YU and BLUMENTHAL (1967) and HAUST (1979) also suggested that elastic fibers were crystal seeders. However, the vesicle hypothesis arising from our observation does not exclude the possibility of other mechanisms in pathological or ectopic mineralization. In contrast, no direct mineralization within or on collagen fibrils and elastic fibers of matrices could be detected in the present culture system.

In conclusion, the results of the present study of ectopic mineralization in cultured fibroblasts support the hypothesis that the initiation of mineralization and calcification requires a microenvironment delimited by a membrane structure derived from cells.

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