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In vitro mineralization in bovine tooth germ cell cultured with sintered hydroxyapatite

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Unerupted teeth were extracted from the mandibles of calves, and the enamel surfaces containing ameloblasts and cells of papilla layer were scraped off. Cells migrated from the fragments were transferred into culture flasks containing small bars of sintered hydroxyapatite, and the cultures were incubated long-term. Most of the cells showed a fibroblastic morphology, but some of the cells formed an epithelial cell nest. The fibroblastic cells grew steadily, but the epithelial cells showed poor growth. The fibroblastic cells formed multiple cell layers and were embedded in collagenous matrix containing type I trimer collagen. By 3 months, fibrous bands appeared on the surface of the cell layers and surrounded the bars of sintered hydroxyapatite. Such bands increased in size with incubation time. Mineral deposition was observed in the well-developed bands. The deposited crystals were found by the X-ray powder diffraction method to be hydroxyapatite; the Ca/P molar ratio of 1.49 was determined by the ICP method. These results indicated that cells of papilla layer have the capacity to mineralize *in vitro* in the presence of sintered hydroxyapatite.

1. Introduction

Tooth enamel is formed by ameloblasts derived from the oral epithelial tissue. In enamel mineralization, the ameloblasts are classified into the secretory and maturation stages. Ameloblasts at the maturation stage are surrounded by dental papilla layer. Culture of the tooth germs was investigated by the organ culture method and the outgrowing cells form dental pulpa and papillae [1–4]. Mineral deposition has been demonstrated in a few cell culture systems of osteoblastic cell lines [5] and primary cultures of calvaria cells and periodontal ligament cells [6–8], but not yet for ameloblasts and cells of the papilla layer. This paper describes primary culture of maturation ameloblasts and cells of the papilla layer derived from bovine tooth germs and *in vitro* mineralization in the outgrowing cells cultured with sintered hydroxyapatite. Mineral deposits were characterized by electron microscopy, X-ray powder diffractometry and chemical analysis.

2. Experimental procedure

2.1. Primary culture

Unerupted incisor teeth were aseptically extracted from the mandibles of 1 year old calves, and the dental sacks were removed. Some of the enamel surfaces were stained with GBHA. Several red strips appeared on the enamel surfaces, confirming ameloblasts to be at the maturation stage [9]. After rinsing with Hanks' balanced salt solution (BSS) supplemented with 500 IU ml^{-1} penicillin and 500 $\mu g\,ml^{-1}$ streptomycin sulphate, the surface layers of the enamel containing ameloblasts and cells of the papilla layer were carefully scraped off. The fragments were placed centrally in 35 mm plastic culture dishes (Falcon, USA), and cultured with alpha modification of Eagle's minimum essential medium (α-MEM) (Gibco, USA) supplemented with 10% foetal bovine serum (Gibco) at 37 °C in a humidified atmosphere of 5% CO_2 in air.

Cells migrated from the fragments were allowed to grow for 7 days. The outgrowing cells were subcultured with 0.05% pronase E (Kaken Seiyaku, Tokyo, Japan) in phosphate buffer saline (PBS(−)). The cells were transferred every week into 5 ml fresh medium in 25 cm^2 culture flasks (Falcon) at 2×10^5 cells/flask. For morphological observation, some cultures were fixed with methanol and stained with Giemsa's solution.

2.2. Extraction and preparation of culture media

Culture media and cells at the primary cultures and the first and sixth passages were examined biochemically by the electrophoresis methods to detect enamel protein and collagen. The cells were dialysed overnight against 0.5 M acetic acid at 4 °C. The media were centrifuged at 5000 g for 30 min and lyophilized. Samples were then analysed by 7.5% SDS-PAGE [10]. To assess the presence of reducible collagen

chains, samples were submitted to interrupted SDS-PAGE [11] in the presence of 10% 2-mercaptoethanol. Samples were electrophoresed using the Tris-glycine system, and gels were stained with Coomassie blue. The presence of amelogenin, enamelin, fibronectin and collagen type I, II and III was further examined by the immunoblotting method [12] using their antibodies.

2.3. Long-term culture

The cells at the second passage were transferred into 25 cm^2 culture flasks containing bars of sintered hydroxyapatite with dimensions of $1 \times 6 \times 45$ mm^3, and the cultures were incubated long-term. The bars of sintered hydroxyapatite used were prepared by the method described by Akao *et al.* [13]; the sintering temperatures were 1200 °C.

The cell morphology and appearance of mineral deposition were frequently observed under a phase-contrast microscope. At 3 months, some cultures were fixed with methanol and stained with 1% alizarin red S solution. At 5 and 7 months, the cultures were fixed with a mixture of 2% paraformaldehyde and 2% glutaraldehyde in 0.05 M phosphate buffer. They were removed mechanically from the flasks, post-fixed with 1% osmium tetraoxide in the buffer, embedded in epoxy resin, sectioned with diamond knives and stained with uranyl acetate and lead citrate for electron microscopy.

An X-ray powder diffraction pattern of the 7 month culture after fixation with methanol was taken with CuK_α radiation at 40 kV and 40 mA, using a silicon single crystal specimen holder which does not exhibit X-ray diffraction. Mineral deposits in the 7 month culture was heated at 800 °C for 1 h, and a Ca/P molar ratio of the residual ash was determined by the inductively coupled plasma (ICP) method.

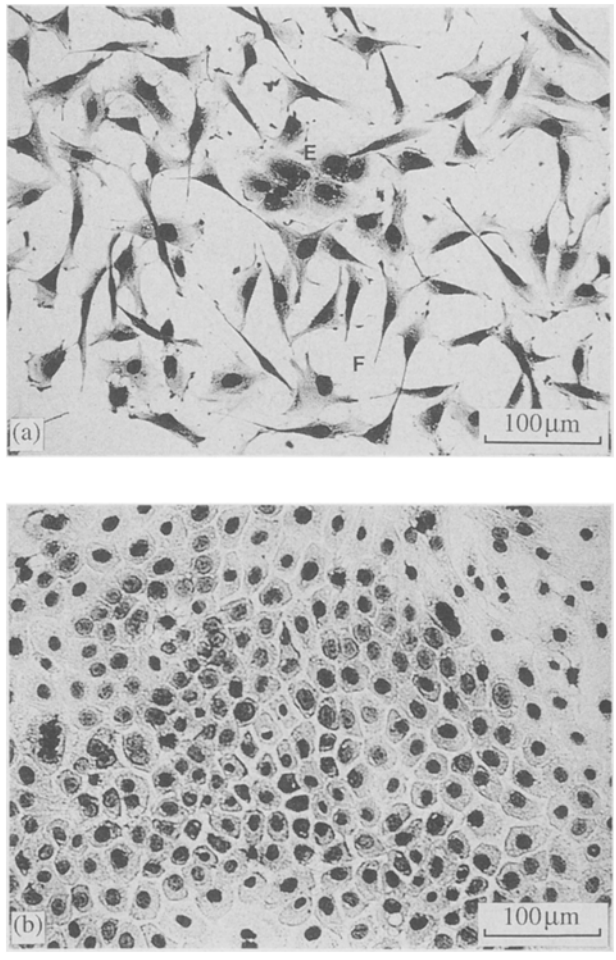


Figure 1 Light microscopy of bovine tooth germ cells in 5 day primary culture stained with Giemsa's solution. (a) Outgrowing cells are a mixed population of fibroblastic (F) cells from the papilla layer and epithelial (E) cells from the ameloblast layer. (b) Epithelial cell nest showing a polygonal morphology.

3. Results

3.1. Primary culture and cell passage

The cells growing out of the fragments were frequently observed at days 3 and 4. They were a mixed population of fibroblastic cells and epithelial cells. Most of the cells showed a fibroblastic morphology (Fig. 1a), but some of the cells showed a polygonal morphology and were closely in contact with each other, forming epithelial cell nests (Fig. 1b). The fibroblastic cells grew steadily, but the epithelial cells showed poor growth. The fibroblastic and epithelial cells originate from cells of the papilla layer and ameloblasts on the enamel surface, respectively. Fibroblasts of the papilla layer are always attached to the ameloblast layer, and mechanical separation of cells of this layer is difficult.

At 7 days, the fibroblastic cells were isolated with 0.05% pronase E. The remaining epithelial cell nests were removed mechanically with a rubber policeman and transferred in the flasks. Fibroblastic cells grew out from the epithelial cell nests which deformed gradually and disappeared at 2 weeks.

The isolated cells in the logarithmic growth phase were spindle-shaped and polymorphic, and in the confluent stage were arranged in a certain direction. Thus, the cells exhibited a fibroblastic morphology. The doubling time of the fibroblastic cells was about 2 days from the third to the fifth passages. Severe reduction in the growth rate was observed in the ninth passage at 2 months, and then the cell growth was terminated owing to ageing.

3.2. Biochemical analysis of culture media

As a result of SDS-PAGE assay, collagen α1 chains and fibronectins were detected in the whole culture media. Fig. 2 shows collagen α1 chains at the first passage after 7.5% SDS-PAGE. The fibroblastic cells have the capacity to synthesize collagen up to the sixth passage. On the other hand, production of amelogenin [14] and enamelin, which are characteristic proteins of enamel substrate, was hardly observed by the SDS-PAGE and immunoblotting methods.

The collagen chains were analysed by interrupted SDS-PAGE and compared with bone, skin and cartilage collagens. After reduction, α1 chains of type I collagen were found, but α2 (I) and α1 (III) chains were not detected. Under these conditions, α1 (III) chains migrated between α1 (I) and β chains. As a result of immunoblotting assay, fractions of the collagen chains

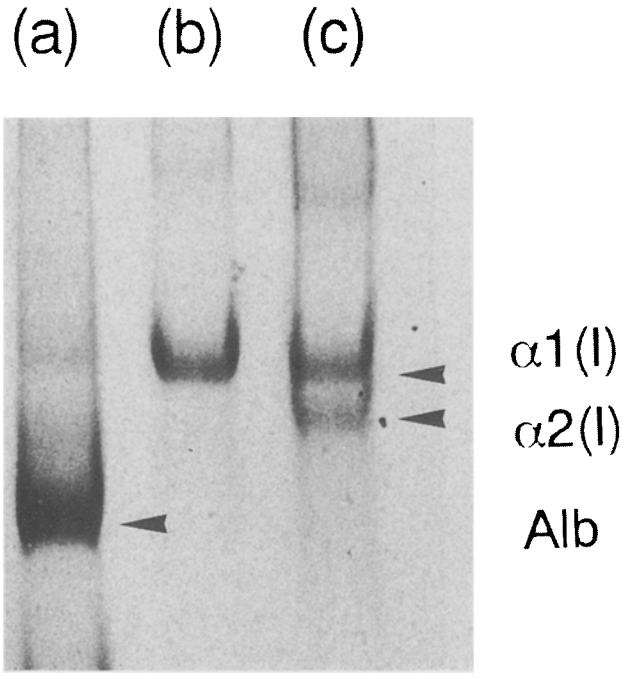


Figure 2 Coomassie blue staining of collagen in (a) the culture medium and (b) extracellular matrix at the first passage after 7.5% SDS–PAGE. (c) Bone collagen served as a control. Alb, serum albumin.

did not bind with anticollagen types I, II and III. These results indicate that the type of collagen expressed in the extracellular matrix is type I trimer, $[\alpha 1(I)]_3$. Production of type I trimer collagen is found in embryonic tendon and calvaria *in vivo* [15] and cultures of dental pulp fibroblasts [2].

3.3. *In vitro* mineralization in long-term culture

In the case of long-term cultures, the cells continued to grow slowly without contact inhibition and formed multiple cell layers. At 3 months, fibrous bands appeared on the surface of the cell layers (Fig. 3a), connecting the bars of sintered hydroxyapatite and the edges of the flasks. Such bands increased in size with incubation time. By 3–5 months, dark striations were observed in the central part of the well-developed bands by phase-contrast microscopy (Fig. 3b). The fibrous bands showed intense staining with alizarin red S, and the dark striations were found to be mineral deposits containing calcium (Fig. 3c). The mineral deposition increased around the bars of sintered hydroxyapatite with incubation time, and became easily identifiable as yellow–brown bands with the naked eye. On the other hand, the cells cultured long-term without sintered hydroxyapatite formed multiple cell layers, but showed lower development of fibrous bands and poor mineralization.

3.4. Electron microscopy

Fig. 4 shows transmission electron microscopy (TEM) of a vertical section of multiple cell layer in a 5 month culture. Several elongated fibroblastic cells were arranged parallel to the surface and were embedded in

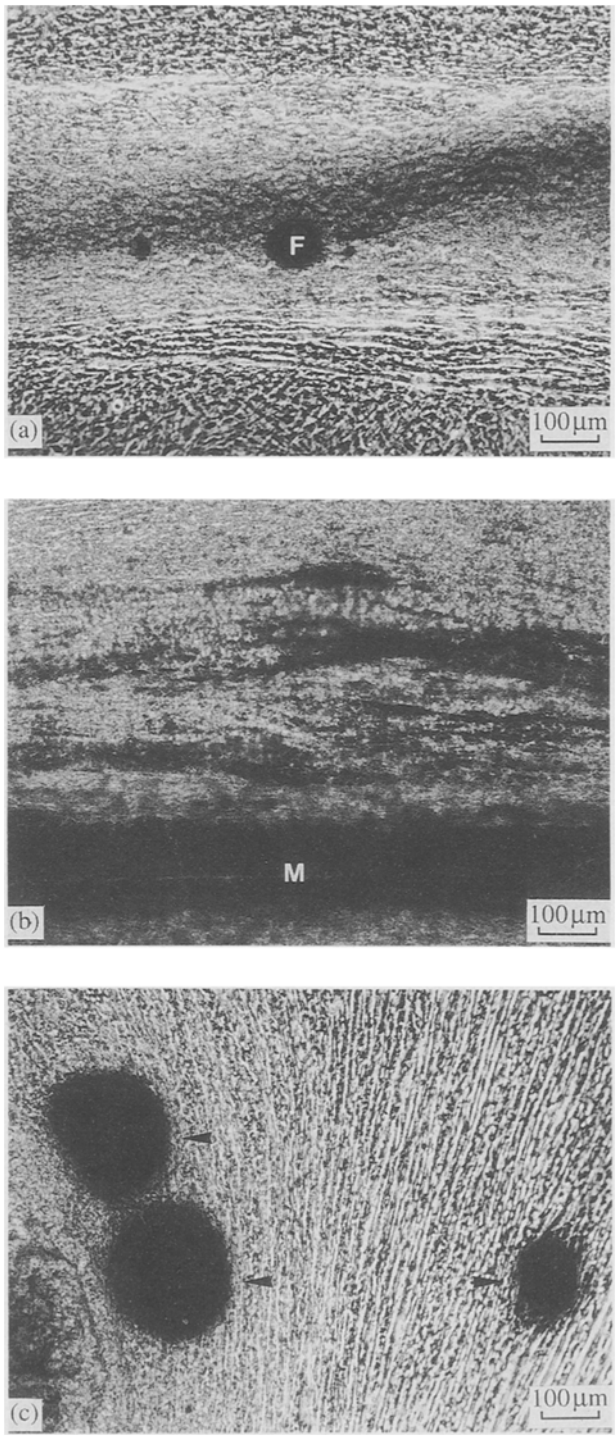
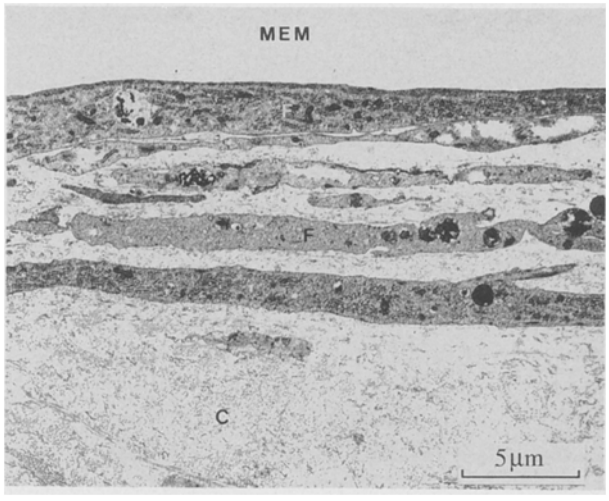


Figure 3 Phase-contrast micrographs of 3 month cultures at the second passage. (a) A fibrous band (F) appeared on the surface of the multiple cell layer stained with alizarin red S. (b) Mineral deposits (M) in the multiple cell layer appear as dark striations. (c) Alizarin red-stained culture showing heavy mineralization (arrowheads) in the well-developed band.

collagenous matrix. As shown in Fig. 5a, the cells had abundant rough endoplasmic reticula and numerous secretory granules. Well-banded collagen fibrils were observed in the extracellular matrix. Thus, the cells showed a morphology of active fibroblasts. The cells were attached to each other by numerous cell processes, the ends or sides of which had gap junctions, desmosomes and tight junctions (Fig. 5a and b). Some of the cells exhibited ultrastructural characteristics of



typical osteoblasts which are rich in mitochondria and Golgi bodies (Fig. 5c).

Mineral deposits were observed among the well-banded collagen fibrils in the 5 month culture and were mainly composed of plate-like crystals (Fig. 6). Axial periodicity of the collagen fibrils ranged from 60–70 nm, which is similar to hard tissue collagen. The mineral deposition among collagen is predominant in the initial stage of mineralization. In the 7 month culture, cells in the heavily mineralized regions

Figure 4 Low-power electron micrograph of a vertical section of the multiple cell layer in a 5 month culture. Elongated fibroblastic (F) cells are embedded in collagenous (C) matrix.

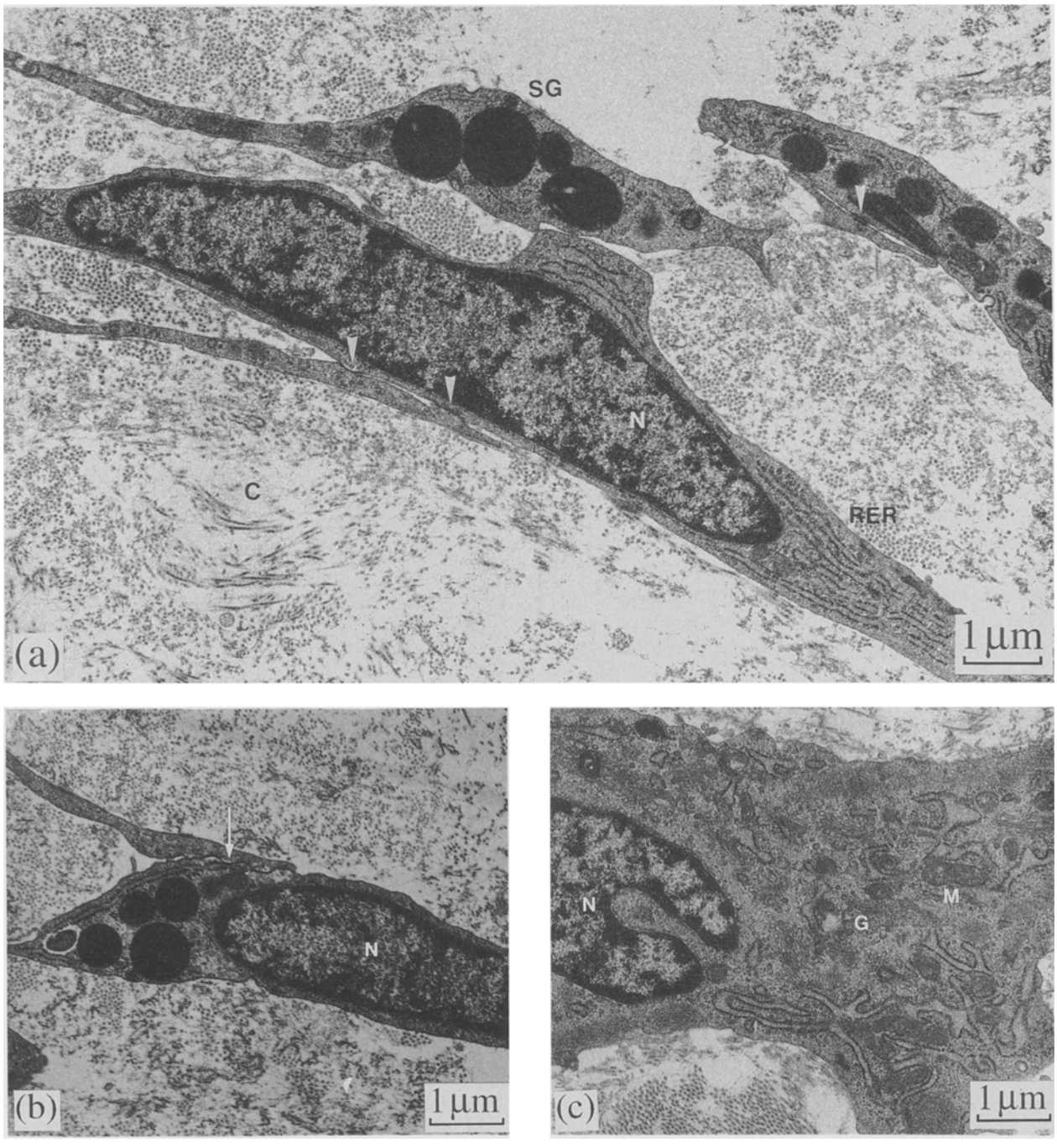


Figure 5 Transmission electron micrographs of 5 month cultures. (a) Fibroblastic cells have abundant rough endoplasmic reticula (RER) and numerous secretory granules (SG). Cells are embedded in collagenous (C) matrix. Desmosomes are indicated by white arrowheads. (b) A tight junction is indicated by a white arrow. (c) Osteoblastic cell rich in mitochondria (M) and Golgi bodies (G).

Figure 6 Mineral deposits (white arrowheads) among well-banded collagen (C) fibrils in a 5 month culture.

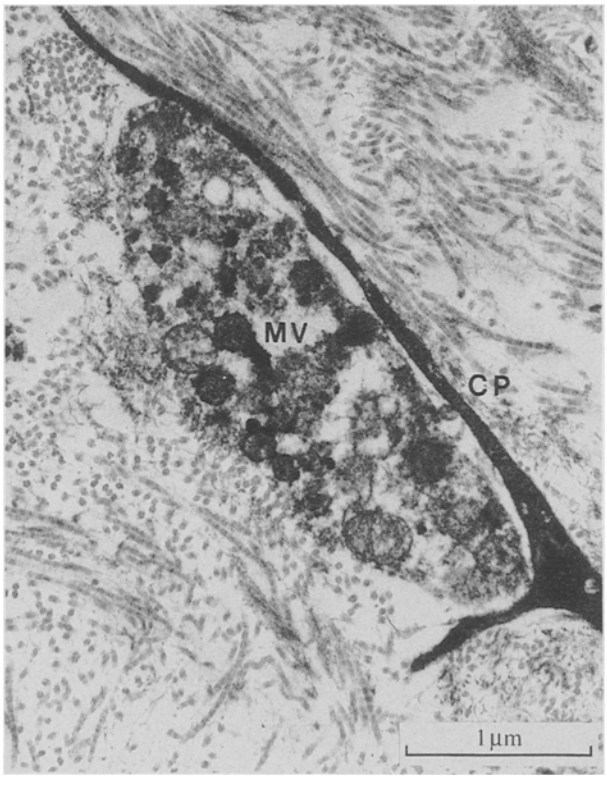


Figure 7 Matrix vesicles (MV) observed around a cell process (CP) in a 7 month culture.

become necrotic. Several matrix vesicles were observed around the cell process (Fig. 7). Double or triple membrane structure of the matrix vesicles was evident. Numerous ultrastructural studies have demonstrated that matrix vesicles play an important role in bone mineralization *in vivo* and *in vitro* [5, 7], but they are rarely observed in acellular cementum *in vivo* and culture of periodontal ligament cells [8]. In the present study, the appearance of matrix vesicles was not so extensive throughout the mineralized regions.

3.5. X-ray powder diffractometry and chemical analysis

Fig. 8 shows an X-ray diffraction pattern of mineral deposits in a 7 month culture. The deposited crystal was identified as hydroxyapatite. The dry weight of the mineral deposits used was 10.9 mg. Table I shows chemical composition of 3.0 mg residual ash heated at 800 °C determined by the ICP method. The Ca/P molar ratio was found to be 1.49. The ratio was much lower than the theoretical value (1.67) of hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$ and near to 1.50 of tricalcium phosphate, $Ca_3(PO_4)_2$.

In the previous studies, mineral deposits were identified by energy-dispersive X-ray (EDX) analysis and electron diffraction patterns [5, 8]. The Ca/P molar ratios reported ranged from 1.0–1.67. The reliability of these data is rather low, because the Os–*M* peak is always incorporated with a P–*K* peak in the EDX analysis. The present results demonstrate that the deposited crystal is calcium-deficient hydroxyapatite.

4. Discussion

The present study clearly demonstrates that cells of the papilla layer have the capacity to differentiate into osteoblasts or cementoblasts and to form mineralized tissue *in vitro* in the presence of sintered hydroxyapatite. *In vitro* formation of mineralized bone-like

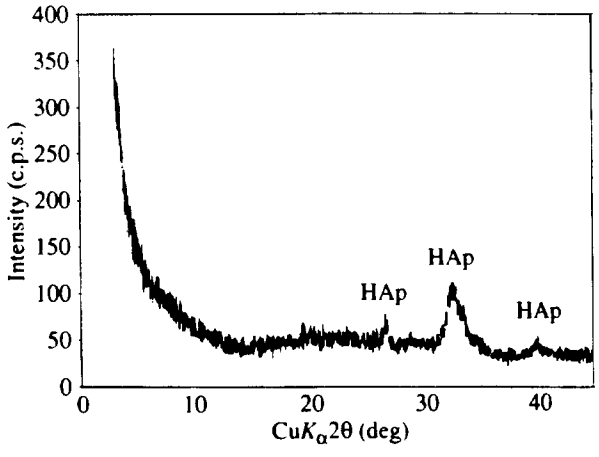


Figure 8 X-ray powder diffraction pattern of mineral deposits in a 7 month culture (CuK_α radiation, 40 kV, 40 mA). HAp, hydroxyapatite.

TABLE I Chemical composition of mineral deposits in a 7 month culture heated at 800 °C

CaO	65.5%
P_2O_5	26.4%
Total	91.9%
Ca/P	1.49

matrix has been intensively studied using osteoblastic cells originating from foetal calvaria [6, 7] and osteoblastic cell lines, MC3T3–E1 [4], ROS 17/2.8 and HOS. It is well known that supplement of β-glycerophosphate to these osteoblastic cell cultures as a source of organic phosphate is required for the successful extracellular mineralization [6]. It has also been reported that supplement of dexamethasone seems to stimulate differentiation into osteoblasts and mineralization in these culture systems [6, 8]. The formation of mineralized tissue in the present study occurred when the cells were cultured with sintered hydroxyapatite in the absence of β-glycerophosphate or dexamethasone.

It has been clearly shown, based on numerous animal experiments, that hydroxyapatite has remarkable compatibility compared to other materials. When sintered hydroxyapatite is implanted in bone tissue, the activation of osteoblasts occurs which can induce the newly formed bone. Cellular activity of osteoblasts and fibroblasts in the presence of hydroxyapatite powders has been tested *in vitro*. Osteoblastic MC3T3–E1, ROS 17/2.8 and calvaria cells are shown to phagocytoze the hydroxyapatite particles, and exhibit reduced cell growth and alkaline phosphatase (ALP) activity [16]. ALP is an enzyme which might be related to mineralization, and a high ALP activity is detected in the early stage of mineralization. In contrast, human pulp fibroblasts cultured with hydroxyapatite powders exhibit increasing ALP activity [4]. MC3T3–E1 cells cultured on the surfaces of sintered hydroxyapatite exhibit increasing cell growth and ALP activity [17]. Thus, *in vitro* cellular response against hydroxyapatite depends on the cell types and culture systems. The present study suggests that sintered hydroxyapatite can induce differentiation into osteoblasts or cementoblasts and mineralization *in vitro*.

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