SHORT COMMUNICATION

Mineralized bone nodule formation *in vitro* by cell populations from young adult rabbit alveolar bone

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Introduction

In vitro mineralization by cells isolated from bone has been studied¹⁻⁶⁾ previously in order to understand the intracellular events and extracellular processes occurring on initiation of bone formation. However, some of the previous protocols for isolating bone cell populations using collagenase solution were rather complicated^{1,2,5)}, and detection of bone nodules (mineral deposits) formed by the isolated cells was time-consuming¹⁻⁵⁾. With regard to oral tissue, little has been reported on the *in vitro* mineralization capacity of cells derived from the mandible⁶⁾. Accordingly, the present study was performed with a view to establishing a simple and reproducible cell culture system using alveolar bone from young adult rabbits, and to demonstrate the capacity of these cells for *in vitro* mineralization in short-term culture.

Materials and Methods

Alveolar bone from young adult rabbit (3 months old) mandible was aseptically fragmented and washed twice with phosphate-buffered saline. The bone fragments were lightly treated with a collagenase mixture, as described previously by Bellows *et al.*²⁾. The enzyme solution was then discarded, and the enzyme-treated bone fragments were cul-

tured for 2 weeks in alpha-minimum essential medium containing 10 % fetal bovine serum and antibiotics (alpha-MEM) in a humidified atmosphere (95 % air/5 % CO₂) at 37°C. The cells growing out of the bone fragments were plated at a density of 2×10^4 /35-mm dish, and then cultured for about 7 days in the medium until they reached confluency. At this point, the culture medium was changed to a supplemented medium containing 50 µg/ml ascorbic acid, 10 mM Na beta-glycerophosphate and 10 nM dexamethasone in alpha-MEM, as described by Tenenbaum *et al.*⁷⁾. The first day was designated as day 0 and the cells were cultured up to day 21; the supplemented medium was changed every second day. Cell growth and nodule expression were routinely checked by phase-contrast microscopy.

For histochemical detection of mineral deposits, every cultured cell sheet on the 35-mm dish was fixed with 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.35), and stained with 2 % aqueous alizarin red S according to Williams *et al.*³⁾. The cell sheet then examined microscopically.

For morphological observation, some of the aldehyde-fixed 21-day preparations were post-fixed 1 % OsO₄, routinely dehydrated in a graded ethanol series, and embedded *in situ* in Spurr's resin. Sections were cut perpendicular to the culture dish surface. Thick sec-

Fig. 1 Polygonal, osteoblast-like cells growing out of an alveolar bone fragment (BF).

Fig. 2 Alizarin red-stained 21-day culture. Several loci showing mineralization are evident, and the surrounding cells are polygonal.

Fig. 3 Toluidine blue-stained section of 7-day culture. Large and small nodules (arrowheads) are located in a mound-like accumulation of cells.

Fig. 4 Ultrastructure of a cell and calcifying nodule (Nd) in 3-day culture. Cell organelles are well developed. Deposition of needle-shaped crystals is evident in the Nd, which is rich in collagen fibrils.

Fig. 5 Ultrastructure of a cell and a highly calcified nodule (Nd) in 14-day culture.

tions for light microscopy were stained with toluidine blue, and ultra thin sections for transmission electron microscopy were stained with uranyl acetate and lead citrate.

Results

Cell populations derived from the alveolar bone fragments mostly showed a distinct polygonal appearance (Fig. 1), and a similar

morphology was observed in the confluent cultures (day 0) as well as the subsequent cultures. Focal accumulations of the cells, each with a central opaque region, were recognizable by microscopy on day 3, and with the naked eye on day 5. These opaque regions increased in diameter and height, and the associated cells became multilayered after day 14. With alizarin red S, the opaque regions showed moderate to intense staining. The staining was not homogeneous, but several intensely-staining loci were demonstrable within each opaque region (Fig. 2). In toluidine blue-staining sections (Fig. 3), cell accumulations were mound-like and the cells were in close apposition with the well stained material, presumably mineralized masses. In addition to the major mineralized masses, several smaller mineral deposits were also observed (Fig. 3).

Ultrastructurally, cells in the early stage of culture appeared to be in a state of active biosynthesis, i. e., the cells possessed well developed rough endoplasmic reticulum, prominent Golgi apparatus and abundant mitochondria (Fig. 4). Needle-shaped apatite-like crystals were present in the peripheral organic matrix of early calcifying nodules (Fig. 4). After a longer period of culture, these nodules were more highly calcified (Fig. 5).

Discussion

For the identification of osteoblast-like cells, various parameters have been used such as the ability to synthesize osteoblast-associated proteins (type I collagen, osteocalcin, osteonectin, etc.), the presence of alkaline phosphatase activity and a tissue-specific hormone response⁹⁾. However, these biochemical parameters, except for osteocalcin, are not unique to osteoblast-like cells. At present, an osteogenic capacity seems to be the most reliable indicator, since the primary function of osteoblasts is bone formation⁹⁾. Therefore, in this preliminary study, we focused on morphological observation combined with histochemical staining to identify mineral deposits formed by alveolar bone cell populations.

In living cultures, the cells were largely polygonal, and an intimate relationship bet-

ween these cells and the nodules was demonstrated histologically. Calcium deposition in the nodules was revealed with alizarin red S staining, and the presence of needle-shaped apatite-like crystals in the nodules was confirmed by ultrastructural examination. In latestage cultures, the nodules became highly calcified. In addition, well developed cell organelles indicative of active biosynthesis were demonstrated ultrastructurally in the cells of early-stage cultures. Based on these findings, we conclude that the cells used in the present study are osteoblast-like and that the nodules formed by them could be classified as bone.

Recent studies on *in vitro* mineralization have used isolated membranous bone cells^{1,2,5)} or an osteosarcoma cell line⁴⁾. In the present study, we demonstrated that freshly prepared mature bone cell populations have a capacity to form bone nodules *in vitro*, that preparation of the cells is simpler than previously reported methods^{1,2,4,5,8,10)} and that our culture system is reproducible.

The effects of various factors on bone nodule formation have been examined^{7,8,10)}. Tenenbaum *et al.*⁷⁾ and Bellows *et al.*⁸⁾ reported the concentrations of beta-glycerophosphate and dexamethasone that suitable for nodule formation. Based on their data, we added beta-glycerophosphate and dexamethasone to the medium and observed reproducible nodule formation. The immediate effect of dexamethasone is considered to be a stimulation of nodule formation⁸⁾. In our culture system, supplementation with dexamethasone seemed to stimulate nodule formation, as reported by Bellows *et al.*⁸⁾.

Detailed ultrastructural examinations of the initiation and subsequent process of calcification are now in progress. Through the use of this culture system, we hope to examine the effects of various factors involved in osteogenesis and to reveal the mineralization nucleators and calcification dynamics.

References

- 1) Sudo, H., Kodama, H., Amagi, Y., Yamamoto, S. and Kasai, S.: *In vitro* differentiation and calcification in a new clonal osteogenic cell line derived from new bone mouse

- calvaria. *J. Cell Biol.* **96** : 191-198, 1983.
- 2) Bellows, C. G., Aubin, J. E., Heersche, J. N. M. and Antosz, M. E. : Mineralized bone nodules formed *in vitro* from enzymatically released rat calvaria cell populations. *Calcif. Tissue Int.* **38** : 143-154, 1986.
 - 3) Williams, D. C., Boder, G. B., Toomey, R. E., Paul, D. C. and Hillman, C. C. Jr. : Mineralization and metabolic response in serially passaged adult rat bone cells. *Calcif. Tissue Int.* **30** : 233-246, 1980.
 - 4) Nishimoto, S. K., Stryker, W. F. and Nimni, M. E. : Calcification of osteoblast-like rat osteosarcoma cells in agarose suspension cultures. *Calcif. Tissue Int.* **41** : 274-280, 1987.
 - 5) Bhargava, U., Bar-lei, M., Bellows, C. G. and Aubin, J. E. : Ultrastructural analysis of bone nodules formed *in vitro* by isolated fetal rat calvaria cells. *Bone* **9** : 155-163, 1988.
 - 6) Umehara, K. and Kawata, T. : A study of calcification in cultured cells from the human mandible. *Jpn. J. Oral Biol.* **28** : 565-572, 1986 (in Japanese).
 - 7) Tenenbaum, H. C. and Heersche, J. N. M. : Differentiation of osteoblasts and formation of mineralized bone *in vitro*. *Calcif. Tissue Int.* **34** : 76-79, 1982.
 - 8) Bellows, C. G., Aubin, J. E. and Heersche, J. N. M. : Physiological concentrations of glucocorticoids stimulate formation of bone nodules from isolated rat calvaria cells *in vitro*. *Endocrinology* **121** : 1985-1992, 1987.
 - 9) Majeska, R. J. and Rodan, G. A. : Culture and activity of osteoblasts and osteoblast-like cells. [Ed. by Butler, W. T. : The chemistry and biology of mineralization tissue], Ebsco Media, Birmingham, Alabama, pp. 279-285, 1984.
 - 10) Antosz, M. E., Bellows, C. G. and Aubin, J. E. : Biphasic effect of epidermal growth factor on bone nodule formation by isolated rat calvaria cells *in vitro*. *J. Bone Mineral Res.* **2** : 385-393, 1987.