

Osteogenic capacity of cultured human periosteal cells

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We developed a culture system of cells isolated from juvenile human periosteum. The culture consisted of epithelial-like and fibroblast-like cells. Both types of cells had intense alkaline phosphatase activity maintained in subculture. When these cells, loaded into diffusion chambers, were implanted subcutaneously in rats, cartilage tissue was mainly formed and bone was seen scantily. 1α -OH- D_3 and $1,25$ -(OH) $_2$ - D_3 increased the alkaline phosphatase activity and proteoglycan synthesis in the periosteal cells. Calcitonin also stimulated the proteoglycan synthesis, but parathyroid hormone had no effect.

Periosteal osteogenesis has been mainly investigated by histologic analysis following heterotopic transplantation of periosteum (Shepelmann 1913, Burman and Umansky 1930, Ham 1930, Halde- man 1932, Urist and McLean 1952, Urist et al. 1965, Ritsilä et al. 1973, Uddströmer and Ritsilä 1978, Poussa and Ritsilä 1979, Sevitt 1981). Free periosteal grafts from young animals can form bone in the tendon (Burman and Umansky 1930) and in the eye (Urist and McLean 1952). Ritsilä and Alhopuro (1973) reported the formation of membranous bone when free periosteum was transplanted to an experimental defect in the tracheal wall and stressed the importance of environmental factors. However, there is still no agreement regarding the osteogenetic mechanism and the cellular metabolism of periosteum. Therefore, we developed a culture system of periosteal cells isolated from young human periosteum.

With this culture system, we studied the biological properties of periosteal cells in culture, the transplantation of cultured periosteal cells, and the metabolic activity of these cells in response to calcium-regulating hormones.

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Materials and methods

Culture of human periosteal cells. Periosteum (1×1 cm) was obtained from tibiae of 8-12-year-old children during operations, excluding patients with a fracture. Tissues were minced and treated with 0.1 per cent EDTA and 0.2 per cent trypsin at 37° C for 1 h. After washing three times with phosphate-buffered saline (PBS), the minced tissues were digested with 0.2 per cent collagenase (type 1, Sigma) at 37° C for 3 h in a digestion chamber. The isolated cells were washed three times with PBS and counted in a hemocytometer. Totally 1×10^6 cells/ml were inoculated into plastic dishes (Falcon, 35 mm) containing 2 ml of Ham's F-12 medium supplemented with 10 per cent fetal bovine serum (FBS, GIBCO) and antibiotics, and incubated at 37° C in 5 per cent CO₂ in air. The medium was changed every other day.

When the cells became confluent, they were removed from the dishes with 500 protease units/ml of Dispase (Godo Shusei Co., Ltd.) in Ham's F-12 medium and passaged at a concentration of 1×10^5 cells/ml in the same medium. The cultures were fixed in 10 per cent neutral formalin and stained with toluidine blue. Alkaline phosphatase activity was tested by the azo dye method. All the experiments were performed using secondary culture of periosteal cells.

Parathyroid hormone (Protein Research Foundation, Osaka), synthetic eel calcitonin (Toyo Jozo Co., Ltd.), and vitamin D₃ metabolites

(Chugai Pharmaceutical Co., Ltd.) were added to the medium 24 h before the experiments.

Implantation in diffusion chamber in vivo. Diffusion chambers (Millipore Ltd., 0.45 μm membrane filters) were filled with 0.1 ml of cell suspension (10^7 cells/ml) and implanted into the dorsal subcutis of adult rats. The chambers were removed after 4 weeks and transferred to 10 per cent neutral formalin. The specimens were dehydrated in graded ethanol and embedded in paraffin. Sections were stained with hematoxylin eosin for light microscopy.

Alkaline phosphatase activity. For assay of alkaline phosphatase, the cultured medium was removed and washed with ice-cold PBS. Then, the cells were collected with a rubber policeman in 1 ml of distilled water and sonicated for 60 s. The crude homogenate was used as an enzyme source. The measurement of alkaline phosphatase activity was based on the hydrolysis of p-nitrophenylphosphate at 37° C in 0.1 M carbonate buffer, pH 10. The specific activity was expressed as μmoles of phenol per μg protein of cell homogenate per 60 min at 37° C.

Glycosaminoglycan synthesis. The synthesis was monitored by measuring incorporation of $\text{Na}_2^{35}\text{SO}_4$ (Suzuki et al. 1976, Uchida et al. 1978). The cell layer was labeled with $2\mu\text{Ci/ml}$ of $\text{Na}_2^{35}\text{SO}_4$ (94 mCi/mmol, New England Nuclear) for 24 h. After washing with chilled PBS to eliminate free radioactivity, the cells were collected in 1 ml of 0.5 M NaOH and solubilized by incubating for 1 h at 37° C and then neutralized with 1 M HCl. Next, proteolytic digestion by pronase E was carried out at 55° C for 12 h. The glycosaminoglycans were precipitated with 1 per cent cetylpyridinium chloride in 0.02 NaCl and the precipitate was counted in a spectrometer (Aloka). Comparisons of the means were determined by the Student's *t*-test.

Results

After 7-14 days cultivation, two types of cell populations were seen - epithelial-like and fibroblast-like cells. The nodules, as mounds of epithelial-like cells, were formed among fibroblastic monolayer cells (Figure 1). These colonies exhibited strong metachromatic staining with tolui-

dine blue, but the fibroblast-like cells had no metachromasia (Figure 2). On the other hand, both types of cells were positive for alkaline phosphatase (Figure 3). The activity was maintained even in fifth generation cells as intensely as in primary cultured cells.

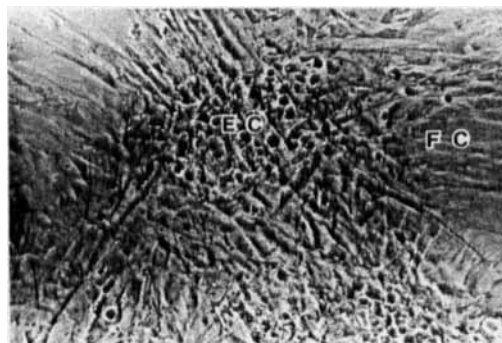


Figure 1. Cultured periosteal cells. Fibroblast-like cells (FC) spread all over the dish, but epithelial-like cells (EC) form some colonies. 14 days of cultivation.

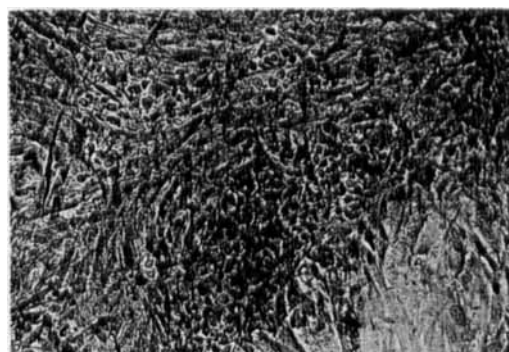


Figure 2. Cultured periosteal cells after 14 days of cultivation. Metachromasia was found in the colonies of epithelial-like cells. Toluidine blue staining, x100.

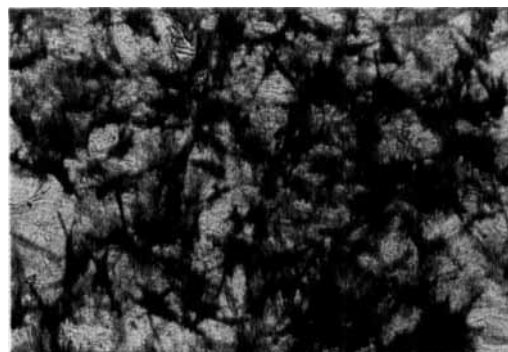


Figure 3. Alkaline phosphatase activity of cultured periosteal cells. Enzymatic activity was detected in almost all the cells on the dish after 14 days of cultivation. Naphthol AS method, x100.

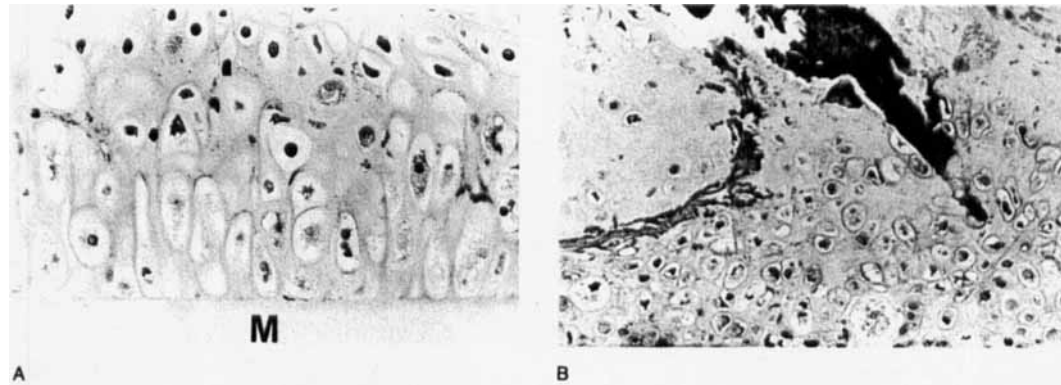


Figure 4. Photomicrograph of cultured periosteal cells transplanted with diffusion chambers into intramuscular region of rats. A. Cartilage tissue was found in contrast to the membrane (M) of a diffusion chamber. B. Woven bone was seen around cartilage.

When the diffusion chambers were removed from the subcutaneous layer after 4 weeks, they were enclosed in a loose fibrous capsule. In the chambers, cartilage tissue was found in contact with the membrane. Hypertrophic chondrocytes were present and arranged in columns in the direction of the membrane. They showed great similarity to the cartilage cells seen in the zone of proliferation and hypertrophy in the growth plate. A small amount of bone was seen around the cartilage tissue, but did not appear to be formed by endochondral ossification (Figure 4), because no newly formed bone was found to follow on the heels of the hypertrophic cartilage cells in a serial section.

Alkaline phosphatase activity of periosteal cells was $2.89 \pm 0.64 \times 10^3 \mu\text{M/h}$ per μg protein in the primary culture. The subcultured cells had similar activity ($2.85 \pm 0.66 \times 10^3 \mu\text{M/h}$ per μg protein).

Parathyroid hormone ($1 \mu\text{g/ml}$) and calcitonin

(100 mu/ml) had no effect on the activity of alkaline phosphatase. On the other hand, vitamin D_3 markedly increased the activity. $1\alpha\text{-OH-D}_3$ and $1,25\text{-(OH)}_2\text{-D}_3$ in 10^{-7} M solution increased the activity by about 80–90 per cent ($P < 0.01$, Table 1). The minimum concentration of D_3 metabolites that elicited a significant increase in the alkaline phosphatase activity in the cells was found to be 10^{-9} M . This stimulatory effect was dose-dependent only at the concentrations of 10^{-9} – 10^{-7} M .

The glycosaminoglycan synthesis of cultured periosteal cells was stimulated by the addition of calcitonin ($100 \text{ m}\mu\text{/ml}$) or both vitamin D_3 metabolites (10^{-7} M) ($P < 0.01$). A not significant slight tendency to an increase in the glycosaminoglycan synthesis by the addition of parathyroid hormone as compared with the matched control was noted. (Table 1).

Table 1. Effects of parathyroid hormone, calcitonin, and D_3 metabolites on alkaline phosphatase activity and glycosaminoglycan synthesis in cultured periosteal cells^a

	Alkaline phosphatase activity ^b ($\times 10^{-3}$) $\mu\text{M/h}\mu\text{g}$ protein)	Per cent of control	³⁵ S uptake ^b (dpm/mg protein)	Per cent of control
Control	2.85 (0.66)		260 (74)	
Parathyroid hormone	2.74 (0.64)	96	362 (48)	139
Calcitonin	2.83 (0.48)	99	781 (135)	293
$1\alpha\text{-OH-D}_3$	5.36 (0.81)	188	572 (118)	220
$1,25\text{-(OH)}_2\text{-D}_3$	5.58 (0.79)	196	603 (103)	232

^a When periosteal cells reached confluency, parathyroid hormone ($1 \mu\text{g/ml}$), calcitonin (100 mu/ml) and D_3 metabolites (10^{-7} M) were added to the medium and cells were harvested 24 h later.

^b M (SE) of results in three experiments.

Discussion

The periosteum comprises an outer fibrous and inner cambium layer. In our culture system, there were also two cell populations – epithelial-like cells and fibroblast-like cells. The former, which have more intense alkaline phosphatase activity and glycosaminoglycan synthesis, seem to be the cells from the cambium layer, which may be osteoblasts, while the fibroblast-like cells, having alkaline phosphatase activity only, appear to be the cells of the outer fibrous layer.

In the healing of a fracture, periosteal cells form fibrous tissue, cartilage, and bone (Byer et al. 1981). Our experiments showed the same three types of tissues in the diffusion chambers loaded with human periosteal cells. This picture thus mimics the one seen during fracture healing. Which factors cause the differentiation of periosteal cells into fibroblasts, chondroblasts or osteoblasts? Oxygen content, electromechanical forces, and other microenvironments around the cells are thought to play important roles in the differentiation process. Our experimental system is a useful tool for studying cell differentiation of periosteum.

Alkaline phosphatase activity in the periosteal cells of rat long bone is strong under normal conditions (Shedden et al. 1976), and rabbit

periosteal cells in culture still maintain high activity of this enzyme (Bitz et al. 1981). Periosteal cells synthesize hardly any proteoglycan in a normal physiologic state, but they are expected to display the ability to undergo proteoglycan synthesis under specific conditions, such as fracture and electrical stimulation. Therefore, alkaline phosphatase activity and proteoglycan synthesis in these periosteal cells under culture are thought to be appropriate indicators of cell metabolism.

Little is known about the effects of calcium-regulating hormones on the metabolism of the periosteum of human long bones. Delling et al. (1969) stated that calcitonin accelerates the rate of healing in early stages and the formation of a periosteal callus. We suggest that calcitonin regulates the process of fracture healing by stimulating proteoglycan synthesis in the periosteal cells.

It is interesting that the D₃ metabolites, whose effect on bone tissue is inconclusive, stimulated alkaline phosphatase activity and proteoglycan synthesis in the periosteal cells. Vitamin D has been found to stimulate the differentiation of the osteogenic cell layer of the periosteum of guinea-pigs during fracture healing (Grauer 1932). Moreover, Lindholm and Sevastikoglou (1978) suggested that 1 α -OH-D₃ and 1,25-(OH)²-D₃, may have effects on the fracture as it heals initially by formation of periosteal callus.

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