

## Effects of Conditioned Media from Periodontal Ligament Cells and Gingival Fibroblasts on the Proliferation and Differentiation in Osteoblastic Clone MC3T3-E1 Cells

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To determine whether the human periodontal ligament cell-conditioned medium (PDL-CM) and the human gingival fibroblast-conditioned medium (GF-CM) affect the proliferation and differentiation of osteoblastic clone MC3T3-E1 cells, the effects of both conditioned media on [<sup>3</sup>H]thymidine incorporation into DNA and alkaline phosphatase (ALP) activity in clone MC3T3-E1 cells were examined. Both PDL-CM and GF-CM increased [<sup>3</sup>H]thymidine incorporation into DNA whereas decreased ALP activity in dose-dependent manner. Especially, their inhibitory effect on ALP activity was proportional to the seeding density of MC3T3-E1 cells and concentration of CM. These findings indicated that PDL and GF produce and release certain soluble factor(s) that stimulate cell proliferation and inhibit differentiation in MC3T3-E1 cells and suggest that PDL and GF may play a role in the regulation of bone cell function *in vivo* as well.

**Key words:** conditioned medium, periodontal ligament cells, gingival fibroblasts, MC3T3-E1, cell proliferation, alkaline phosphatase activity.

### Introduction

Bone cell function is regulated by systemic factors such as steroids, vitamin D<sub>3</sub>, parathyroid hormone and calcitonin, and also, by local factors including cytokines and growth factors produced and secreted by osteogenic and non-osteogenic cells. The earlier studies of regulation of bone cell were limited to the effects of systemic factors, but these studies did not permit definitive assignment of the effects observed to a particular cell type; and the need for local modifications of these messages to develop the predictable shape of the skeleton was not amenable to experimentation.

Recently, the local regulation of bone cell function has been studied extensively providing us a much broader perspective of the activities and possibilities for bone cells. Although a great deal is known about factors that may influence the synthetic activity of osteogenic cells, there is much less information on paracrine regulation of osteogenesis by non-osteogenic cells. These interrelationships between osteogenic cells and nonosteogenic cells *in vivo* can be seen in cranial sutures during growth and development (Evans, 1978), in the periodontal li-

gament (Vignery and Baron, 1980) and in pathological situations such as incomplete healing of fractures of long bones (Ham and Harris, 1971). In these situations, it appears that soft connective tissue cells occupy spatially defined domains that are maintained by local inhibition of osteogenesis. Selective killing of these cells results in bony ingrowth into the space occupied by the soft connective tissue (Line *et al.*, 1974). *In vitro* studies have reported that human gingival fibroblasts stimulated calcium release from mouse calvarial organ culture (Lerner and Hanstrom, 1978), human periodontal ligament cells inhibited parathyroid hormone-stimulated calcium release from rat long bone organ cultures (Giniger *et al.*, 1991), and human periodontal ligament fibroblasts inhibited the formation of mineralized bone nodules in rat bone marrow stromal cells (Ogiso *et al.*, 1991). But bone marrow cultures have various cell populations and organ cultures of bone represent quantum leaps in complexity from isolated cells with respect to both cell populations and intercellular interactions. Thus, ascribing observed functions to a particular cell type is often hazardous. To know the precise effect of certain agents or drugs, it would be desirable to separate a par-

ticular cell type from other cells.

Recently, Kodama *et al.* (Kodama *et al.*, 1982) isolated osteoblastic clone MC3T3-E1 cells from newborn mouse calvaria. These cells retain the ability to produce bone-liver-kidney type alkaline phosphatase activity and Type I collagen and to mineralize ground substance (Sudo *et al.*, 1983; Kumegawa *et al.*, 1984). They also respond to prostaglandin E<sub>2</sub> and PTH (Hakeda *et al.*, 1985) and have a specific receptor for Vitamin D<sub>3</sub> (Kurihara *et al.*, 1986).

In the present study, the effects of the human periodontal ligament cell-conditioned medium (PDL-CM) and the human gingival fibroblast-conditioned medium (GF-CM) on the proliferation and differentiation in osteoblastic clone MC3T3-E1 cells were investigated.

## Materials and methods

### Materials

Media, fetal bovine serum (FBS) and other cultural reagents were obtained from GIBCO laboratories (Grand Island, NY, USA) and plastic culture wares from Nunc (Roskilde, Denmark). [<sup>3</sup>H]thymidine was purchased from Amersham (Arlington Heights, IL, USA). All other reagents were, unless otherwise stated, purchased from Sigma (St. Louis, MO, USA).

### Cell cultures

MC3T3-E1 cells, obtained from Harvard School of Dental Medicine were cultured in  $\alpha$ -minimum essential medium ( $\alpha$ -MEM) supplemented with 15% FBS, 50  $\mu$ g/ml ascorbic acid, 10 mM  $\alpha$ -glycerophosphate, 10<sup>-7</sup> M dexamethasone, 100  $\mu$ g/ml penicillin, 50  $\mu$ g/ml gentamycin, 0.3  $\mu$ g/ml amphotericin B and incubated at 37°C in 95% humidified air plus 5% CO<sub>2</sub>.

Human periodontal ligament cells were isolated from periodontal ligament explants of impacted third molars of healthy adults between 20 and 25 years, as previously described (Mariotti and Cochran, 1990).

Human gingival fibroblasts were isolated from in-

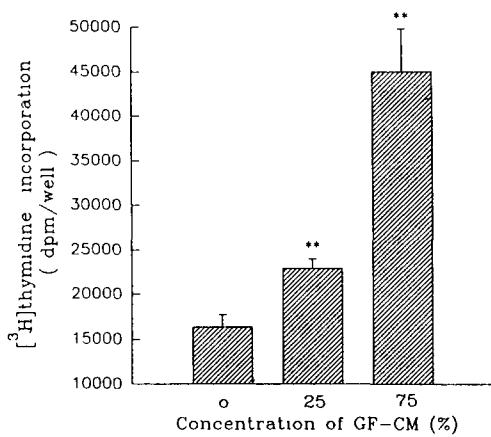
terproximal premolar or molar gingival papilla of clinically healthy tissue from adults (20 to 25) in a similar fashion. Cultures were maintained in  $\alpha$ -MEM containing 10% FBS and media were changed every 2 days. Conditioned media (CMs) from human periodontal ligament cells and human gingival fibroblasts were collected from cultures between the 5th and the 8th passage, and was stored at -20°C until used for experiments. CMs were reconstituted with supplements as for MC3T3-E1 cell cultures above and stored at 4°C prior to use. CMs were added individually to MC3T3-E1 culture medium to final concentration of 25% or 75%.

### Cell proliferation

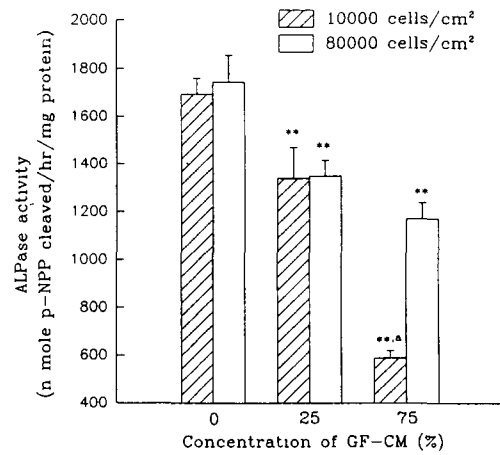
As an index of cell proliferation, DNA synthesis rate was evaluated by measuring the incorporation of [<sup>3</sup>H]thymidine into trichloroacetic acid (TCA)-insoluble fraction. MC3T3-E1 cells were plated into 24 well plates (growth area; 1.9 cm<sup>2</sup>) at a seeding density of 8 × 10<sup>4</sup> cells/cm<sup>2</sup> and were treated with each CM for 24 hrs. During the final 4 hrs, cells were pulsed with 5  $\mu$ Ci/ml of [<sup>3</sup>H] thymidine and incorporation was terminated by aspiration of medium. Cells were then fixed for 10 min at 4°C and washed 4 times with ice-cold 5% TCA. Precipitated radioactivity was solubilized in 0.5 M NaOH and measured with liquid scintillation counter (Beckman, LS 500 TA).

### Alkaline phosphatase activity

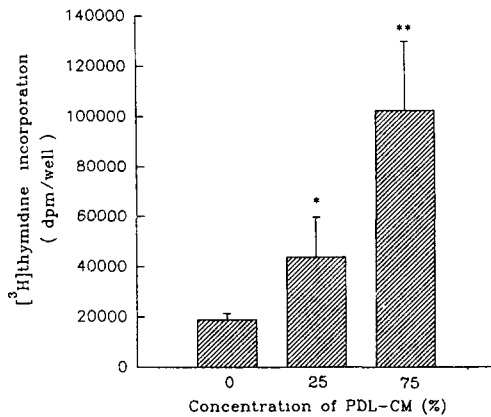
Alkaline phosphatase activity was measured by spectrophotometry using p-nitrophenyl phosphate (15 mM) as substrate. MC3T3-E1 cells were plated into 35 mm culture dishes (growth area; 9 cm<sup>2</sup>) at the density of 1 × 10<sup>4</sup> cells/cm<sup>2</sup> or 8 × 10<sup>4</sup> cells/cm<sup>2</sup> and were treated with each CM for 72 hrs. CM-treated cells were collected in 0.5 ml distilled water and sonicated. Aliquots of the cell homogenate were incubated at 37°C for 30 min in 0.1 M glycine-NaOH buffer (pH 10.3). The optical density of p-nitrophenol, a reaction product, was read at 410 nm using spectrophotometer (Bausch and Lomb, Spetronic 21). Total protein content in cell homogenate was determined by method of Lowry *et al.*



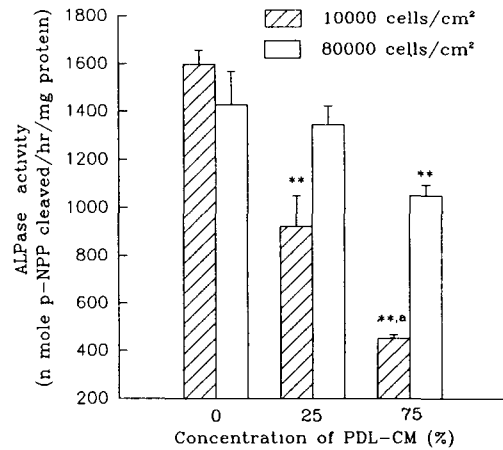
**Fig. 1.** Effect of gingival fibroblast-conditioned medium (GF-CM) on  $[^3\text{H}]$ thymidine incorporation in cultures of MC3T3-E1 cells. Cultures maintained at density of  $8 \times 10^4$  cells/cm<sup>2</sup> into 24-well plates were treated with GF-CM for 24 hrs, pulsed with 5  $\mu\text{Ci/ml}$  of  $[^3\text{H}]$ thymidine during the final 4 hrs and incorporated radioactivity into TCA-insoluble fraction was measured. Bars represent mean S.D. for n=4.  
GF-CM; gingival fibroblast-conditioned medium  
\*\*P<0.01, significantly different from control.



**Fig. 3.** Effect of gingival fibroblast-conditioned medium (GF-CM) on the alkaline phosphatase activity in cultures of MC3T3-E1 cells. Alkaline phosphatase activity was measured by spectrophotometry using p-nitrophenyl phosphate (15 mM) as a substrate. MC3T3-E1 cells were plated into 35 mm culture dishes (growth area; 9 cm<sup>2</sup>) at density of  $1 \times 10^4$  cells/cm<sup>2</sup> or  $8 \times 10^4$  cells/cm<sup>2</sup> and were treated with CM for 72 hrs. Bars represent mean S.D. for n=4.  
\*\*P<0.01, significantly different from control.  
\*P<0.01, vs. culture plated with  $8 \times 10^4$  cells/cm<sup>2</sup>.



**Fig. 2.** Effect of periodontal ligament cell-conditioned medium (PDL-CM) on  $[^3\text{H}]$ thymidine incorporation in cultures of MC3T3-E1 cells. Cultures maintained at density of  $8 \times 10^4$  cells/cm<sup>2</sup> into 24-well plates were treated with PDL-CM for 24 hrs, pulsed with 5  $\mu\text{Ci/ml}$  of  $[^3\text{H}]$ thymidine during the final 4 hrs and incorporated radioactivity into TCA-insoluble fraction was measured. Bars represent mean S.D. for n=4.  
PDL-CM; periodontal ligament cell-conditioned medium  
\*P<0.05 and \*\*P<0.01, significantly different from control.



**Fig. 4.** Effect of periodontal ligament cell-conditioned medium (PDL-CM) on the alkaline phosphatase activity in cultures of MC3T3-E1 cells. Alkaline phosphatase activity was measured by spectrophotometry using p-nitrophenyl phosphate (15 mM) as a substrate. MC3T3-E1 cells were plated into 35 mm culture dishes (growth area; 9 cm<sup>2</sup>) at density of  $1 \times 10^4$  cells/cm<sup>2</sup> or  $8 \times 10^4$  cells/cm<sup>2</sup> and were treated with CM for 72 hrs. Bars represent mean S. D. for n=4.  
\*\*P<0.01, significantly different from control.  
\*P<0.01, vs. culture plated with  $8 \times 10^4$  cells/cm<sup>2</sup>.

(Lowry *et al.*, 1951) with bovine serum albumin as a standard.

## Results

### Effect of Conditioned Medium on Cell Proliferation

### feration

Both PDL-CM and GF-CM significantly stimulated proliferation in clone MC3T3-E1 cells in dose-

dependent manner as demonstrated by [<sup>3</sup>H]thymidine incorporation assay (Fig. 1, Fig. 2). At concentration of 25% or 75% GF-CM, DNA synthesis was 1.4- and 2.8-fold greater than that of control cultures respectively. With PDL-CM at both concentration, 2.3- and 5.4-fold increases compared to control were noted.

#### **Effect of Conditioned Medium on Alkaline Phosphatase Activity**

Both PDL-CM and GF-CM significantly decreased ALP activity per mg protein in dose-dependent fashions (Fig. 3, Fig. 4). Especially, at high concentration (75%) of CMs and low seeding density of cells, CMs exhibited a greater inhibitory effect.

### **Discussion**

Both GF-CM and PDL-CM increased cell proliferation whereas both CMs decreased ALP activity. Especially, both CMs had a greater inhibitory effect on ALP activity at low seeding density of cells and high concentration of CMs.

These results revealed that stimulatory effects on proliferation were accompanied by inhibitory effects on differentiation as determined by cytochemical parameters and can have three possible explanations: first, CMs have main effect on cell proliferation and relatively small number of remaining differentiated cells in the presence of CMs results in decreased ALP activity, especially at low seeding density. But in our experiments, the growth rates of cells were very low (about 10<sup>4</sup> dpm/well). So, it can be thought that the population of proliferating cells may be very small and have little effects on the change of ALP activity in, at least, high seeding group; second, one can postulate that proliferation and differentiation are independently regulated. This is supported by time course experiments employing epidermal growth factor (Antosz *et al.*, 1987) and transforming growth factor- $\beta$  in the culture of osteoblastic MC3T3-E1 (Noda and Rodan, 1986). In this model, it is possible that CM has various soluble factors which stimulate cell proliferation but inhibit differenti-

ation; and lastly, it has been recently postulated that proliferation is functionally related to the synthesis of a bone-specific extracellular matrix. The maturation and organization of the extracellular matrix contribute to the down-regulation of proliferation, which then promotes expression of genes that render the matrix competent for mineralization (Lian and Stein, 1993). In this model, proliferation is intimately related to the differentiation. This relationship is supported by a series of studies in which cells were cultured at various concentrations of ascorbic acid (Owen *et al.*, 1990). These working hypothesis provides a possibility that suppression on ALP activity precludes down-regulation of proliferation.

It has been reported that responses of osteoblasts appear to depend upon a number of variables, such as species differences, cell density, and proliferative state, all of which reflect the stage of differentiation of the cells being examined (Rodan and Rodan, 1984; Chen and Feldman, 1981). Majeska and Rodan (1982) showed that the degree of differentiation and expression of the osteoblast phenotype of an osteoblastlike cell clone (ROS 17/2.8) depends on the seeding density of these cells in culture. When these cells were seeded at low density, they showed a low degree of differentiation in terms of alkaline phosphatase activity, whereas those that were seeded at high density expressed high alkaline phosphatase activity. Also, MC3T3-E1 cells, nontransformed murine cells, have low levels of ALP when they are subconfluent, but this ALP activity changes rapidly to increased levels at confluence. Current results showed that both CMs had a greater inhibitory effect on ALP activity at low seeding density (subconfluent stage) than that at high seeding density (confluent stage). These findings reflect that inhibitory effect of CMs is specific action on the early stages of differentiation process rather than a generalized one over the several stages in developmental sequence of MC3T3-E1 cells.

### **Conclusion**

The present study was undertaken to investigate the effects of conditioned media prepared from hu-

man periodontal ligament cells and human gingival fibroblasts on the proliferation and differentiation in clone MC3T3-E1 cells. It has been shown that both PDL-CM and GF-CM stimulated cell proliferation and also inhibited differentiation as determined by [<sup>3</sup>H]thymidine incorporation and alkaline phosphatase activity. In addition, the current study has shown that both CMs have greater inhibitory effects on ALP activity at low seeding density of cells. Current results lead to speculate that a selective inhibitory effect of CMs on the early stages of differentiation precludes the down-regulation of proliferation. These observations suggest that human periodontal ligament cells and human gingival fibroblasts may regulate the function of osteoblasts *in vivo* as well.

## References

- Antosz, M.E., Bellows, C.G. and Aubin, J.E. : Biphasic effects of epidermal growth factor on bone formation by isolated rat calvaria cells *in vitro*. *J. Bone Miner. Res.* **3**: 185-194, 1987.
- Chen, T.L. and Feldman, D. : Regulation of 1,25-dihydroxyvitamin D<sub>3</sub> receptors in cultured mouse bone cells. *J. Biol. Chem.* **256**: 5561-5566, 1981.
- Evans, C.A. : Facial growth. Section A: Mechanisms. In: *Textbook of Oral Biology*. J.H. Sha, E.A. Sweney, C. C. Cappuccino, and S.M. Meller, eds. W.B. Saunders & Co., Toronto, pp. 55-109, 1978.
- Giniger, M.S., Norton, L., Sousa, S., Lorenzo, J.A. and Bonner, F. : A human periodontal ligament fibroblast clone releases a bone resorption inhibition factor *in vitro*. *J. Dent. Res.* **70**: 99-101, 1991.
- Hakeda, Y., Nakatani, Y., Kurihara, N., Ikeda, E., Maeda, N. and Kumegawa, M. : Prostaglandin E<sub>2</sub> stimulates collagen and non-collagen protein synthesis and prolyl hydroxylase activity in osteoblastic clone MC3T3-E1 cells. *Biochem. Biophys. Res. Commun.* **126**: 340, 1985.
- Ham, A.W. and Harris, W.R. : Repair and transplantation of bone. In: *The Biochemistry and Physiology of Bone*. Vol III, G.H. Bourne, ed. Academic Press, New York, pp. 337-399, 1971.
- Kodama, H., Amagai, Y., Sudo, H., Kasai, S. and Yamamoto, S. : Establishment of a clonal osteogenic cell line from newborn mouse calvaria. *Jpn. J. Oral Biol.* **23**: 899-901, 1982.
- Kumegawa, M., Ikeda, E., Tanaka, S., Haneji, T., Yora, T., Sakagishi, Y., Minami, N. and Hiramatsu, M. : The effects of prostaglandin E<sub>2</sub>, parathyroid hormone, 1,25 dihydroxycholecalciferol, and cyclic nucleotide analogs of alkaline phosphatase activity in osteoblastic cells. *Calcif. Tissue Int.* **36**: 72-76, 1984.
- Kurihara, N., Ishizuka, S., Kiyoki, M., Haketa, Y., Ikeda, K. and Kumegawa, M. : Effects of 1,25-dihydroxyvitamin D<sub>3</sub> on osteoblastic MC3T3-E1 cells. *Endocrinology* **118**: 940-947, 1986.
- Lerner, V. and Hanstrom, L.: Human gingival fibroblasts secrete nondialyzable, prostanoid-independent products which stimulate bone resorption *in vitro*. *J. Periodont. Res.* **22**: 284-289, 1987.
- Lian, J.B., Stein, G.S., Owen, T.A., Bortell, R., Shalhoub, V., Bidwell, J., Breen, E., Barone, L.M., van Wijnen, A. and Aronow, M.: Regulation of the bone cell phenotype. *The Biological Mechanism of Tooth Movement and Craniofacial Adaptation*, Edited by Z. Davidovitch, pp 159-179, 1992, Ohio State University, College of Dentistry, Columbus, Ohio, USA.
- Lian, J.B. and Stein, G.S.: The developmental stages of osteoblast growth and differentiation exhibit selective responses of genes to growth factors (TGF- $\beta$ 1) and hormones (vitamin D and glucocorticoids). *J. Oral Implantol.* **19**: 95-105, 1993.
- Line, S.E., Polson, A.M. and Zander, H.A.: Relationship between periodontal injury, selective cell repopulation and ankylosis. *J. Periodont.* **45**: 725-730, 1974.
- Lowry, O.H., Roseborough, N.D., Farr, A.L. and Rano, R.I.: Protein measurement with the Folin Phenol reagent. *J. Biol. Chem.* **193**: 265-275, 1951.
- Majeska, R.J. and Rodan, G.A.: The effect of 1,25 (OH)<sub>2</sub>D<sub>3</sub> on alkaline phosphatase in osteoblastic osteosarcoma cells. *J. Biol. Chem.* **257**: 3362-3365, 1982.
- Mariotti, A. and Cochran, D.L.: Characterization of fibroblast derived from human periodontal ligament and gingiva. *J. Periodont.* **61**: 103-111, 1990.
- Noda, M. and Rodan, C.A.: Type- $\beta$  transforming growth factor inhibits proliferation and expression of alkaline phosphatase in murine osteoblast-like cells. *Biochem. Biophys. Res. Commun.* **140**: 56-65, 1986.
- Ogiso, B., Hughes, F.J., Melcher, A.H. and McCulloch, C.A.G. : Fibroblasts inhibit mineralized bone nodule formation by rat bone marrow stromal cells *in vitro*. *J. Cell. Physiol.* **146**: 442-450, 1991.
- Owen, T.A., Aronow, M., Shalhoub, V., Barone, L.M., Wilming, L. and Tassinari, M.S. : Progressive development of the rat osteoblast phenotype *in vitro*: Reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. *J. Cell. Physiol.* **143**: 420-430, 1990.
- Rodan, G.A. and Rodan, S.B.: Expression of the osteoblastic phenotype. In: *Bone and Mineral Research*, Vol. 2. W.A. Peck, ed. Elsevier, Amsterdam, pp. 244-

- 285, 1984.
- Sudo, H., Kodama, H., Amagai, Y., Yamamoto, S., and Kasai, S.: *In vitro* differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. *J. Cell Biol.* **96**: 191-198, 1983.
- Vignery, A. and Baron, R.: Dynamic histomorphometry of alveolar bone remodelling in the adult rat. *Anat. Res.* **196**: 191-200, 1980.