

## EFFECTS OF SEVERAL CYTOKINES ON THE FUNCTIONS OF FETAL RAT OSTEOBLAST-LIKE CELLS IN VITRO

Hee-Sung Han, D.D.S., M.S.D., Ph.D., Jung-Keun Kim, D.D.S., M.S.D., Ph.D.,  
Young-Il Chang, D.D.S., M.S.D., Ph.D.

Effects of several cytokines (IL-1 $\beta$ , TNF $\alpha$ , and IFN $\gamma$ ) have been examined on fetal rat osteoblast-like cells. To investigate whether cytokines play direct causal roles in production of lysosomal enzyme, fetal rat osteoblast-like cells were treated with IL-1 $\beta$ , TNF $\alpha$ , and IFN $\gamma$ , respectively or combined.

And acid phosphatase was determined by biochemical method. Alkaline phosphatase was assayed to determine the effects of IL-1 $\beta$ , TNF $\alpha$ , and IFN $\gamma$  on the expression of this enzyme. And also experiment of calcified nodule formation was performed to assess the effects of cytokines on the bone-forming activity of osteoblast-like cells in vitro.

Acid phosphatase activity was significantly increased by the addition of IL-1 $\beta$  and TNF $\alpha$ , whereas decreased by IFN $\gamma$ . However, no significant changes in alkaline phosphatase activity was observed when the osteoblast-like cells were treated with IL-1 $\beta$  and TNF $\alpha$ . Interestingly, IFN $\gamma$  showed stimulatory effect on alkaline phosphatase activity.

The number of calcified nodules was decreased by treatment of cultures with 1 ng/ml IL-1 $\beta$ , 20 ng/ml TNF $\alpha$ , and 500 u/ml IFN $\gamma$  continuously for 21 days, while considerable number of calcified nodules were formed in control group of osteoblast-like cell in culture for 21 days.

These results seem to suggest that cytokines may play crucial roles in bone remodeling through the direct action on the osteoblast-like cell.

**Key words :** Osteoblast-like cell, Interleukin-1 $\beta$ , Tumor necrosis factor  $\alpha$ , Interferon  $\gamma$ , Phosphatase, Bone remodeling

The objective of orthodontic treatment is to produce an occlusion which is healthy and functionally satisfactory, aesthetically satisfactory and stable<sup>16</sup>. Accurate and precise control of tooth movement can be optimized with the proper use of mechanics and knowledge of the subsequent tissue response. Although two comprehensive reviews<sup>11,29</sup> on the mechanism of tooth movement suggest that the controversies related to the histology of tooth movement are near resolution, the mechanisms for conversion of orthodontic force into cellular activity are not completely understood.

Force-induced tooth movement is facilitated by resorption of alveolar bone, a cell-mediated process that presumably may be regulated by factors derived from the nervous and immune systems<sup>5</sup>. Neurotransmitters and cytokines play a regulatory role in

orthodontic force-induced alveolar bone remodeling<sup>5</sup>. Dewhirst et al.<sup>6</sup> recently reported that in fetal rat long bones in vitro, IL-1 and PTH, which respectively stimulated only a small degree of bone resorption, caused extensive resorption when both were present in low concentrations.

Although the effector cell of bone resorption is the osteoclast, studies demonstrating that osteoblasts and not osteoclasts exhibit receptors for parathyroid hormone, vitamin D metabolites, and prostaglandins indicate that osteoclast recruitment and activity involve cells of the osteoblast lineage<sup>24</sup>. Heath et al.<sup>15</sup> reported that osteoblasts responded to systemic and local resorbing agents by producing collagenase, and Chamber et al.<sup>2,3</sup> showed that osteoclasts in vitro do not resorb bone unless the surface osteoid layer is first removed, either by the action of collagenase or

by a layer of osteoblasts. It seems that the critical role of the osteoblast in facilitating bone resorption probably involves low molecular weight osteoblastic signals which activate osteoclasts<sup>22)</sup>.

Cytokines have been shown to be strongly pro-inflammatory *in vivo*, and its release from a number of cell types affects on various biological functions. Many cells including epithelial cells, connective tissue cells, neuronal cells, and leukocytes have been reported as releasing and containing IL-1 $\beta$ <sup>12,19,21,27)</sup>. And also many cells produce TNF $\alpha$  and/or IFN $\gamma$  and they affect on the variety of cells<sup>1,7,10,17,18,30,31,32,33,37)</sup>.

Although these cytokines have been studied on the osteoclast generation and bone resorption, nothing is yet known about the effects of the cytokines on the osteoblastic functions such as enzyme activity and mineralization. In this study the effects of three cytokines (interleukin-1 $\beta$ , tumor necrosis factor  $\alpha$ , and interferon  $\gamma$ ) on the function of fetal rat osteoblast-like cells are to be shown through the investigations on the acid and alkaline phosphatase activity and calcified nodule formation.

## MATERIALS AND METHODS

### (1) Chemicals

Recombinant human cytokines (IL-1 $\beta$ , TNF $\alpha$ , IFN $\gamma$ ) were purchased from Genzyme (Cambridge, MA). Collagenase, tissue culture media and supplements were obtained from Gibco (Grand Island, NY). All disposable culture wares were purchased from Corning Incorporated (Corning, NY) and chamber slides were obtained from Nunc Inc. (Naperville, IL). Staining kits for alkaline phosphatase and acid phosphatase and all other chemicals were obtained from Sigma Chemical (St. Louis, MO).

### (2) Cells and cell culture

Osteoblastic-enriched cell populations were isolated from 19 to 21-day-old fetal Sprague-Dawley rat calvaria according to the methods described by Woo et al.<sup>38)</sup>. Briefly, rat calvaria were dissected aseptically and digested with enzyme solution containing 0.1% collagenase, 0.05% trypsin and 0.5 mM EDTA. At 10,

10, 10, 20 and 20 minute interval (this procedure yielded five populations of cells designated as I, II, III, IV and V), released cells of populations IV and V, which were characterized as osteoblast-like cells, were washed 2 times with Hank's balanced salt solution (HBSS). The cells were plated in a 60 mm culture dish in minimum essential medium supplemented with 10% fetal bovine serum (MEM/FBS) and incubated for 7 days at 37 °C in 95% humidified air containing 5% CO<sub>2</sub>. After 7 days, the cells were plated in culture dishes or culture plates according to its purposes.

### (3) Assay of acid and alkaline phosphatase

To study the effects of cytokines on the activities of ACP and ALP, osteoblast-like cells were plated at a density of 1.5-4.0 x 10<sup>4</sup> cells/well into 96-microwell culture plates and cultured in MEM. When the cells reached confluency, they were treated with IL-1 $\beta$  (0.1-1.0 ng/ml), TNF $\alpha$  (2-20 ng/ml) and IFN $\gamma$  (50-500 u/ml), and without cytokines as a control. To study the effects of combination treatment on the ACP and ALP activity, the cells were treated with cytokine combinations as follows: 1 ng/ml IL-1 $\beta$  + 20 ng/ml TNF $\alpha$ , 1 ng/ml IL-1 $\beta$  + 500 u/ml IFN $\gamma$ , 20 ng/ml TNF $\alpha$  + 500 u/ml IFN $\gamma$ , 1 ng/ml IL-1 $\beta$  + 20 ng/ml TNF $\alpha$  + 500 u/ml IFN $\gamma$ . Cell viability was assessed by trypan blue dye-exclusion to discern the cytotoxic effect of each concentration of cytokines. After 48 hour of culture, the cells were washed with phosphate-buffered saline (PBS) and disrupted by incubation in 0.1 % triton X-100 /saline for 30 min at room temperature. For the ACP assay, the mixture containing 20  $\mu$ l of cell extract, 20  $\mu$ l of 0.1 M sodium citrate buffer (pH 4.8), and 40  $\mu$ l of p-nitrophenyl phosphate (40 mM in 0.01 M sodium acetate, pH 4.8) as substrate was incubated at 37 °C for 30 min. For the ALP assay, 0.1 M of glycine-NaOH buffer (pH 10.3) was used instead of sodium citrate buffer. The reaction was terminated by the addition of 100  $\mu$ l of 1 M NaOH. The wells were read at 405 nm by spectrophotometer (LST400 SFL). The specific activity per milligram protein was calculated for each sample after determining their protein concentrations by Lowry method<sup>20)</sup>.

**Table 1.** Effect of IL-1 $\beta$  on the acid and alkaline phosphatase activity in cultures of fetal rat osteoblast-like cells

Treatment (IL-1 $\beta$ , ng/ml)	Phosphatase activity( $\mu$ M PNP/h/mg protein)	
	Acid	Alkaline
0(Control)	0.78 $\pm$ 0.06	1.25 $\pm$ 0.11
0.1	0.92 $\pm$ 0.10	1.43 $\pm$ 0.08
0.5	1.18 $\pm$ 0.14*	1.28 $\pm$ 0.16
1	1.54 $\pm$ 0.11**	1.33 $\pm$ 0.06

Data represent Mean $\pm$ SEM of 6 replicates.

\* Significantly different from control, p<0.05

\*\* Significantly different from control, p<0.01

**Table 2.** Effect of TNF $\alpha$  on the acid and alkaline phosphatase activity in cultures of fetal rat osteoblast-like cells

Treatment (TNF $\alpha$ , ng/ml)	Phosphatase activity( $\mu$ M PNP/h/mg protein)	
	Acid	Alkaline
0(Control)	0.78 $\pm$ 0.06	1.25 $\pm$ 0.11
2	0.72 $\pm$ 0.10	1.35 $\pm$ 0.13
10	1.05 $\pm$ 0.15	1.30 $\pm$ 0.08
20	1.36 $\pm$ 0.12**	1.24 $\pm$ 0.06

Data represent Mean $\pm$ SEM of 6 replicates.

\*\* Significantly different from control, p<0.01

#### (4) Calcified nodule experiments

Osteoblast-like cells were plated into 35 mm culture dish at a density of  $4 \times 10^4$  cells/dish and maintained until confluency was reached. Cells were cultured in MEM supplemented with 50  $\mu$ g/ml ascorbic acid and 10 mM  $\beta$ - glycerophosphate, and each cytokine(IL-1 $\beta$ , 1 ng/ml; TNF $\alpha$ , 20 ng/ml; IFN $\gamma$ , 500 u/ml) was added and cultured for 3 weeks with changing the medium every 2 to 3 days. To observe the produced calcified materials, each dish was stained by von Kossa's staining. Dishes were fixed at the end of the culture period with 2 % neutral buffered formalin, stained with the von Kossa technique for calcium mineral deposits and counterstained with toluidine blue for the cell. Mineralized nodules were counted on a grid using a microscope.

**Table 3.** Effect of IFN $\gamma$  on the acid and alkaline phosphatase activity in cultures of fetal rat osteoblast-like cells

Treatment (IFN $\gamma$ , u/ml)	Phosphatase activity( $\mu$ M PNP/h/mg protein)	
	Acid	Alkaline
0(Control)	0.78 $\pm$ 0.06	1.25 $\pm$ 0.11
50	0.85 $\pm$ 0.10	1.32 $\pm$ 0.17
250	0.51 $\pm$ 0.05*	1.75 $\pm$ 0.10*
500	0.41 $\pm$ 0.06**	2.44 $\pm$ 0.17**

Data represent Mean $\pm$ SEM of 6 replicates.

\* Significantly different from control, p<0.05

\*\* Significantly different from control, p<0.01

**Table 4.** Effect of cytokine combination on the acid and alkaline phosphatase activity in cultures of fetal rat osteoblast-like cells

Treatment	Phosphatase activity( $\mu$ M PNP/h/mg protein)	
	Acid	Alkaline
0(Control)	0.85 $\pm$ 0.07	1.33 $\pm$ 0.10
IL-1 $\beta$ +TNF $\alpha$	1.69 $\pm$ 0.15**	1.42 $\pm$ 0.02
IL-1 $\beta$ +IFN $\gamma$	1.27 $\pm$ 0.15*	1.85 $\pm$ 0.08**
TNF $\alpha$ +IFN $\gamma$	0.79 $\pm$ 0.10	1.66 $\pm$ 0.14
IL-1 $\beta$ +TNF $\alpha$ +IFN $\gamma$	1.01 $\pm$ 0.09	1.80 $\pm$ 0.15*

Data represent Mean $\pm$ SEM of 6 replicates.

\* Significantly different from control, p<0.05

\*\* Significantly different from control, p<0.01

#### (5) Statistical analysis

Data were analyzed using Student's t-test. Results are expressed as Means  $\pm$  SEM.

## RESULTS

### (1) Assay of acid and alkaline phosphatase

To study the effects of cytokines on ACP and ALP activities, cells were treated with each cytokine single or combination manner. Acid phosphatase activity was significantly increased by the addition of IL-1 $\beta$  and dosedependently, whereas alkaline phosphatase activity was not changed by any concentration of IL-1 $\beta$ (Table 1). 1 ng/ml IL-1 $\beta$  stimulated the acid phosphatase activity by 2-fold compared with control

**Table 5.** Effects of cytokines on the calcified nodule formation of fetal rat osteoblast-like cells

Group	Relative Percentage of calcified nodule <sup>a</sup>
None(control)	100
IL-1 $\beta$ (1ng/ml)	13.3 $\pm$ 2.6 <sup>b**</sup>
TNF $\alpha$ (20ng/ml)	23.7 $\pm$ 5.4 <sup>b**</sup>
IFN $\gamma$ (500U/ml)	60.2 $\pm$ 8.4 <sup>b*</sup>

<sup>a</sup> Values expressed as percent of control.<sup>b</sup> mean  $\pm$  SEM of 3 experiments.\* Significantly different from control,  $p < 0.01$ \*\*Significantly different from control,  $p < 0.001$ **Table 6.** Lack of cytotoxicity of cytokines on rat osteoblast-like cells

Cytokine	Concentration	Relative cell No. (% of control)
Control		100 $\pm$ 6
IL-1 $\beta$ (ng/ml)	0.01	101 $\pm$ 4
	0.1	98 $\pm$ 4
	1	101 $\pm$ 8
TNF $\alpha$ (ng/ml)	0.2	96 $\pm$ 4
	2	107 $\pm$ 7
	20	103 $\pm$ 6
IFN $\gamma$ (U/ml)	5	103 $\pm$ 5
	50	100 $\pm$ 7
	500	97 $\pm$ 7

Data are Mean  $\pm$  SEM

group. TNF $\alpha$  also increased acid phosphatase activity as dose-dependent manner from 2 to 20 ng/ml of TNF $\alpha$ . However, no significant changes in alkaline phosphatase activity was observed when the osteoblast-like cells were treated with TNF $\alpha$  (Table 2). It is of the interest that IFN $\gamma$  affected on both enzyme activities; acid and alkaline phosphatase. IFN $\gamma$  showed inhibitory effect on acid phosphatase activity dose-dependently, whereas it stimulated alkaline phosphatase activity (Table 3). Table 4 shows the effects of cytokine combination on the acid and alkaline phosphatase activity. The acid phosphatase activities of osteoblast-like cells treated with combination of IL-1 $\beta$ +TNF $\alpha$  and IL-1 $\beta$ +IFN $\gamma$  were still higher than that of control, even though those did not showed synergistic effects. However, the stimulatory effect of TNF $\alpha$  on acid phosphatase activity seemed to

**Table 7.** Lack of cytotoxicity of cytokine combination on rat osteoblast-like cells

Cytokine combination	Relative cell No. (% of control)
Control	100 $\pm$ 4
IL-1 $\beta$ (1ng/ml) + TNF $\alpha$ (20ng/ml)	94 $\pm$ 7
IL-1 $\beta$ (1ng/ml) + IFN $\gamma$ (500U/ml)	99 $\pm$ 6
TNF $\alpha$ (20ng/ml) + IFN $\gamma$ (500U/ml)	102 $\pm$ 7
IL-1 $\beta$ + TNF $\alpha$ + IFN $\gamma$	95 $\pm$ 6

Data are Mean  $\pm$  SEM

be abolished by IFN $\gamma$  action. The increased activities of alkaline phosphatase induced by IFN $\gamma$  generally were inhibited either by IL-1 $\beta$  or TNF $\alpha$ .

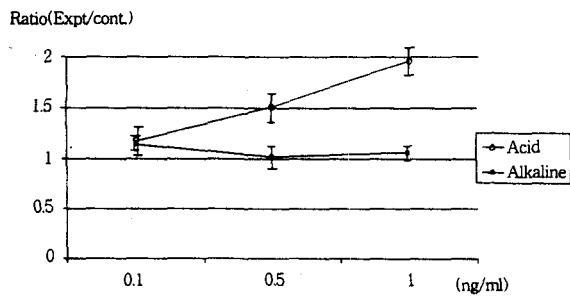
## (2) Calcified nodule formation

The number of calcified nodules was decreased by treatment of cultures with 1 ng/ml IL-1 $\beta$ , 20 ng/ml TNF $\alpha$ , and 500 u/ml IFN $\gamma$  continuously for 21 days, while considerable number of calcified nodules were formed in control group of rat osteoblast-like cells in culture for 21 days. IL-1 $\beta$  and TNF $\alpha$  was significantly different from control ( $p < 0.001$ ) and IFN $\gamma$  was significantly different from control ( $p < 0.01$ ), (Table 5).

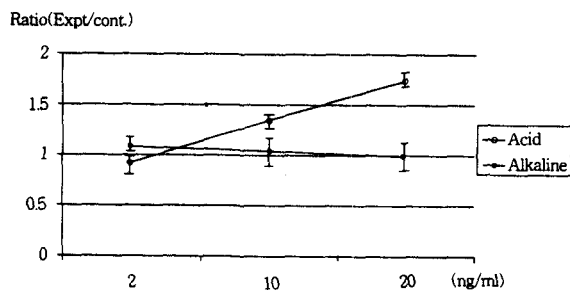
## DISCUSSION

Within the concentrations used in this experiment, any concentration of cytokines did not affect on the cell proliferation. Assessment of cell viability by trypan blue-exclusion revealed no discernible cytotoxic effect for the treatment of 3 cytokines: IL-1 $\beta$  (-1 ng/ml), TNF $\alpha$  (-20 ng/ml), and IFN $\gamma$  (-500u/ml) (Table 6 and 7).

Interleukin-1 (IL-1) is a multifunctional cytokine which is one of the crucial molecules of the body defence mechanism to microbial invasion, inflammatory and immunologic reactions, and tissue injury. In our assay of acid and alkaline phosphatase activity, IL-1 $\beta$  (0.5 and 1 ng/ml) was able to stimulate the acid phosphatase activity without a change in the alkaline phosphatase activity (Figure 1). As reviewed by Tatakis<sup>36)</sup>, IL-1 $\beta$  stimulates the synthesis of prostaglandins and thromboxane that have been



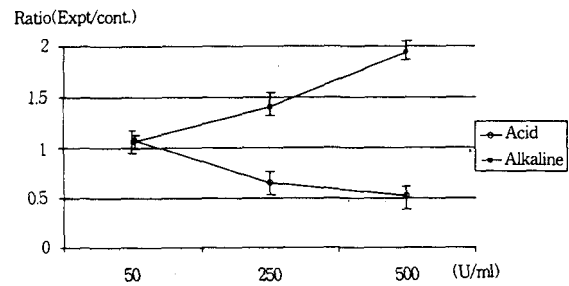
**Fig 1.** Effect of IL-1 $\beta$  on the acid and alkaline phosphatase activity in cultures of fetal rat osteoblast-like cells



**Fig 2.** Effect of TNF $\alpha$  on the acid and alkaline phosphatase activity in cultures of fetal rat osteoblast-like cells

known as bone-resorbing agents and induces the production of collagenase from the rheumatoid synovial cells<sup>25)</sup>. The number of calcified nodule was diminished markedly by the treatment of IL-1 $\beta$  to the osteoblast-like cells which had been maintained for 21 days with ascorbic acid(50  $\mu$ g/ml) and  $\beta$ -glycerophosphate(10 mM). This result is in accordance with the previous studies by Stashenko et al.<sup>34)</sup> and Nguyen et al.<sup>26)</sup> in that IL-1 $\beta$  inhibits the bone formation in vitro.

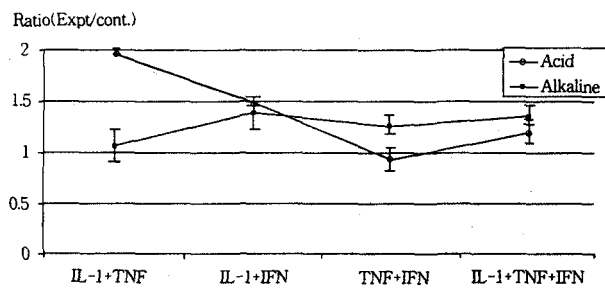
TNF $\alpha$  has been shown to induce the secretion of collagenase, prostaglandin E<sub>2</sub>, and IL-6 by human fibroblasts and bone cells<sup>4,8,23)</sup>. As shown in Figure 2, TNF $\alpha$  also stimulated acid phosphatase activity without a significant change of alkaline phosphatase. It has been reported similarly by Klebanoff et al.<sup>18)</sup> that neutrophils are stimulated to release the lysosomal enzymes by TNF $\alpha$ . It suppressed the formation of calcified nodule when the osteoblast-like cells were treated with 20 ng/ml of TNF $\alpha$  for 21 days(Table 5). Taken together the results of IL-1 $\beta$  and TNF $\alpha$ , these two cytokines showed similar effects



**Fig. 3.** Effect of IFN $\gamma$  on the acid and alkaline phosphatase activity in cultures of fetal rat osteoblast-like cells

on the acid and alkaline phosphatase activity and calcified nodule formation in cultures of osteoblast-like cells. These findings not only support the hypothesis that these cytokines could involve the bone tissue destruction and inhibition of bone formation, so called bone remodeling, but they also raise the question of what cells are actually produced and respond locally to IL-1 $\beta$  and TNF $\alpha$  in the tissues, as well as how these cytokines interact with other immune mediators in bone tissue.

Interferon is heterogeneous family of multifunctional cytokines whose first demonstrated biological activity was the induction of cellular resistance to viral infection. However, their actions on cell growth and differentiation and their many immunoregulatory activities are of great interest in biological and clinical significance. IFN $\gamma$  has been shown to be a potent inhibitor of IL-1 $\beta$ , TNF $\alpha$ , parathyroid hormone, and vitamin D-stimulated bone resorption in vitro<sup>9,13,28)</sup>. IFN $\gamma$  also inhibits bone resorption by inhibition of recruitment and fusion of precursors for multinucleated or osteoclast-like cells in mouse and human marrow cultures<sup>35)</sup>. In this study, it is of great interest that IFN $\gamma$  stimulated alkaline phosphatase and inhibited acid phosphatase significantly(Figure 3). Although the number of calcified nodule is less than the control, the number and size are much bigger than the groups treated with IL-1 $\beta$  and TNF $\alpha$ . In this study, increased activity of alkaline phosphatase did not affect on the calcified nodule formation, even if the expression of alkaline phosphatase is an early event in osteogenic differentiation. The size of calcified nodules formed by long-term culture of osteoblast-like cells were smaller and more particulated than the control



**Fig. 4.** Effect of cytokine combination on the acid and alkaline phosphatase activity in cultures of fetal rat osteoblast-like cells.

when the cells were treated with IL-1 $\beta$  or TNF $\alpha$ . However, IFN $\gamma$  did not affect on the calcified nodule size (Table 5).

In the experiment of cytokine combinations, IFN $\gamma$  showed inhibitory effect on the acid phosphatase stimulated by IL-1 $\beta$  and TNF $\alpha$  (Figure 4). This inhibitory effects of IFN $\gamma$  on the IL-1 $\beta$  and TNF $\alpha$  can be in accordance with the reports concerning to the osteoclastic bone resorption induced by several cytokines and osteotropic hormones<sup>9,14,31</sup>.

It can be concluded from the present study that IL-1 $\beta$  and TNF $\alpha$  increase the acid phosphatase activity and inhibit the mineralization without any influence on alkaline phosphatase activity. Whereas IFN $\gamma$  inhibits the acid phosphatase activity and stimulates alkaline phosphatase activity. These results seem important for understanding the direct actions of cytokines in osteoblastic function and bone remodeling.

## CONCLUSIONS

The present study was performed to investigate whether cytokines play direct roles in the osteoblastic activity. And acid and alkaline phosphatase was assayed to determine the effects of IL-1 $\beta$ , TNF $\alpha$ , and IFN $\gamma$  on the expression of these enzymes by biochemical method in cultures of fetal rat osteoblast-like cells. And also experiment of calcified nodule formation was performed to assess the effects of cytokines on the bone-forming activity of osteoblast-like cells in vitro.

The results are as follows ;

1. Acid phosphatase activity was significantly in-

creased by the addition of IL-1 $\beta$  and TNF $\alpha$ , whereas decreased by IFN $\gamma$ .

2. No significant changes in alkaline phosphatase activity was observed when the osteoblast-like cells were treated with IL-1 $\beta$  and TNF $\alpha$ .
3. IFN $\gamma$  showed stimulatory effect on alkaline phosphatase activity.
4. The number of calcified nodules was decreased by treatment of cultures with 1 ng/ml IL-1 $\beta$ , 20 ng/ml TNF $\alpha$ , and 500 u/ml IFN $\gamma$ .

## Author Address

**Hee-Sung Han**, D.D.S., M.S.D., Ph.D.

*Private Practice in Han Wool Dental Clinic,  
Hogae-Dong 1046-1, Dongan-Ku, Anyang,  
430-080, Korea*

*Tel) 82-343-87-2587*

*Fax) 82-343-87-2589*

*Department of Orthodontics, College of Dentistry,  
Seoul National University.*

*Yeounkun-Dong 28, Chongno-Ku, Seoul,  
110-749, Korea*

*Tel) 82-2-760-2678*

**Jung-Keun Kim**, D.D.S., M.S.D., Ph.D.

*Department of Biochemistry, College of Dentistry,  
Dankook, University.*

*Anseo-Dong San 29, Chunan, 330-714, Korea*

*Tel) 82-417-550-1926*

*Fax) 82-417-552-7646*

## REFERENCES

1. Beutler, B., Greenwald, D., Hulmes, J.D., Chang, M., Pan, Y.-C.E., Mathison, J., Ulevitch, R. and Cerami, A. : Identity of tumor necrosis factor and the macrophage-secreted factor cachectin, *Nature*, 316 : 552-554, 1985
2. Chambers, T.J., Darby, J.A. and Fuller, K. : Mammalian collagenase predisposes bone surfaces to osteoclastic resorption, *Cell Tissue Res.*, 241 : 671-675, 1985.
3. Chambers, T.J. and Fuller, K. : Bone cells predispose bone surfaces to resorption by exposure of mineral to osteoclastic contact, *J. Cell. Sci.*, 76 : 155-165, 1985.
4. Chaudhary, L.R., Spelsberg, T.C. and Riggs, B.L. : Production of various cytokines by normal human

- osteoblast-like cells in response to interleukin-1 $\beta$  and tumor necrosis factor- $\alpha$ : lack of regulation by 17 $\beta$ -Estradiol, *Endocrinology*, 130 : 2528-2534, 1992.
5. Davidovitch, Z., Nicolay, O.F., Ngan, P.W. and Shanfeld, J.L. : Neurotransmitters, cytokines and the control of alveolar bone remodeling in orthodontics, *Dent. Clin. North Am.*, 32 : 411-435, 1988.
  6. Dewhirst, F.E., Ago, J.M. and Peros, W.J. : Synergism between parathyroid hormone and interleukin 1 in stimulating bone resorption in organ culture, *J. Bone Min. Res.*, 2 : 127-134, 1987.
  7. Djeu, J.Y., Blanchard, D.K., Halkias, D. and Friedman, H. : Growth inhibition of *Candida albicans* by human polymorphonuclear neutrophils : activation by interferon gamma and tumor necrosis factor, *J. Immunol.*, 137 : 2980-2984, 1986.
  8. Elias, J.A., Gustilo, K., Baeder, W. and Freundlich, B. : Synergistic stimulation of fibroblast prostaglandin production by recombinant interleukin 1 and tumor necrosis factor, *J. Immunol.*, 138 : 3812-3816, 1987.
  9. Fujii, Y., Sato, K., Kasono, K., Satoh, T., Fujii, T. and Shizume, K. : Prolonged and ubiquitous inhibition by interferon- $\gamma$  of bone resorption induced by parathyroid-related protein, 1,25-dihydroxy vitamin D<sub>3</sub> and interleukin 1 in fetal mouse forearm bones, *Calcif. Tissue Int.*, 47 : 178-182, 1990.
  10. Garrett, R., Durie, B.G.M., Nedwin, G.B., Gillespie, A., Bringman, T., Sabatini, M., Bertolini, D.R. and Mundy, G.R. : Production of lymphostatin, a bone-resorbing cytokine, by cultured human myeloma cells, *N. Engl. J. Med.*, 317 : 526-532, 1987.
  11. Gianelly, A.A. and Goldman, H.M. : Biologic basis of orthodontics, pp116-204, Lea & Febiger, Philadelphia, 1971.
  12. Giulian, D., Baker, T.J., Shin, L.C.N. and Lachman, L.B. : Interleukin 1 of the central nervous system is produced by ameboid microglia, *J. Exp. Med.*, 164 : 594-604, 1986.
  13. Gowen, M., Nedwin, G.E. and Mundy G.R. : Preferential inhibition of cytokine-stimulated bone resorption by recombinant interferon gamma, *J. Bone Miner. Res.*, 1 : 469-474, 1986.
  14. Gowen, M., Wood, D.D., Ihrle, E.J., Meats, J.E and Russel, R.G.G. : Stimulation by human interleukin 1 of cartilage breakdown and production of collagenase and proteoglycanase by human chondrocytes but not by human osteoblasts in vitro, *Biochim. Biophys. Acta.*, 797 : 186-193, 1984.
  15. Heath, J.K., Atkinson, S.J., Meikle, M.C. and Reynolds, J.J. : Mouse osteoblasts synthesize collagenase in response to bone resorbing agents, *Biochim. Biophys. Acta.*, 802 : 151-154, 1984.
  16. Houston, W.J.B. : Orthodontic diagnosis, Dental practitioner handbook, pp91-94, 3rd ed., Wright PSG Inc. Bristol, London, Boston, 1982.
  17. Kehrl, J.H., Miller, A. and Fauci, A.S. : Effect of tumor necrosis factor alpha on mitogen activated human B cells, *J. Exp. Med.*, 166 : 786-791, 1987
  18. Klebanoff, S.J., Vadas, M.A., Harlan, J.M., Sparks, L.H., Gamble, J.R., Agosti, J.M. and Waltersdorff, A.M. : Stimulation of neutrophils by tumor necrosis factor, *J. Immunol.*, 136 : 4220-4225, 1986.
  19. Lovett, D.H. and Larsen, A. : Cell cycle-dependent interleukin 1 gene expression by cultured glomerular mesangial cells, *J. Clin. Invest.*, 82 : 115-122, 1988.
  20. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. : Protein measurement with folinphenol reagent, *J. Biol. Chem.*, 193 : 265-275, 1951.
  21. Luger, T.A., Stadler, B.M., Katz, S.I. and Oppenheim, J.J. : Epidermal cell (keratinocyte)-derived thymocyte-activating factor(ETAF), *J. Immunol.*, 127 : 1493-1498, 1981.
  22. Mcsheehy, P.M.J. and Chambers, T.J. : Osteoblast-like cells in the presence of parathyroid hormone release soluble factor that stimulates osteoclastic bone resorption, *Endocrinology*, 119 : 1654-1659, 1986.
  23. Meikle, M.C., Atkinson, S.J., Ward, R.V., Murphy, G. and Reynolds, J.J. : Gingival fibroblasts degrade type 1 collagen films when stimulated with tumor necrosis factor and interleukin 1, *J. Periodontal Res.*, 24 : 207-213, 1989.
  24. Meikle, M.C., Heath, J.K., Atkinson, S.J., Hembry, R.M. and Reynolds, J.J. : Molecular biology of stressed connective tissue at sutures and hard tissue in vitro, In : Norton, L.A. and Burstone, C.J. ed. *The biology of tooth movement*, pp71-86, CRC Press, Inc Boca Raton, Florida, 1989.
  25. Murphy, G., Reynolds, J.J. and Werb, J. : Biosynthesis of tissue inhibitor of metalloproteinase by human fibroblasts in culture : stimulation by 12-O-tetradecanoyl-phorbol 13-acetate and interleukin 1 in parallel with collagenase, *J. Biol. Chem.*, 260 : 3079-3083, 1985.
  26. Nguyen, L., Dewhirst, F.F., Hauschka, P.V., Stashenko, P. : Interleukin-1 $\beta$  stimulates bone resorption and inhibits bone formation in Vivo, *Lymphokine Cytokine Res.*, 10 : 15-21, 1991.
  27. Ollivierre, F., Gubler, U., Towle, C.A., Laurencin, C. and Treadwell, B. V. : Expression of IL-1 genes in human and bovine chondrocytes : a mechanism for autocrine control of cartilage matrix degradation, *Biochem. Biophys. Commun.*, 141 : 904-911, 1986.
  28. Peterlik, M., Hoffmann, O. Swetly, P., Klaushofer, K. and Koller, K. : Recombinant gamma-interferon inhibits prostaglandin-mediated and parathyroid hormone-induced bone resorption in cultured neonatal mouse

- calvaria, *FEBS Lett*, 185 : 287-290, 1985.
29. Reitan, K. : Biomechanical principles and Reactions, In : Graber, T.M. and Swain, B.F. : Current orthodontic concepts and technique, pp111-229, ed. 2, W.B. Saunders Company, Philadelphia, 1975.
  30. Sato, K., Kasono, K., Fujii, Y., Kawakami, M., Tsushima, T. and Shizume, K. : Tumor necrosis factor type alpha (cachectin) stimulates mouse osteoblast-like cells (MC3T3-E1) to produce macrophage-colony stimulating activity and prostaglandin E2, *Biochem. Biophys. Res. Commun.*, 145 : 323- 329, 1987.
  31. Sato, K., Satoh, T., Shizume, K., Yamakawa, Y., Ono, Y., Demura, H., Akatsu, T., Takahashi, N. and Suda, T. : Prolonged decrease of serum calcium concentration by murine  $\gamma$ -Interferon in hypercalcemic, human tumor (EC-GI)-bearing nude mice, *Cancer Res.*, 52 : 444-449, 1992.
  32. Shalaby, M.R., Aggarwal, B.B., Rinderknecht, E., Svedersky, L.P., Finkle, B.S. and Palladino, M.A. Jr. : Activation of human polymorphonuclear neutrophil functions by interferon-gamma and tumor necrosis factors, *J. Immunol.*, 135 : 2069-2073, 1985.
  33. Stashenko, P., Dewhirst, F.E., Peros, W.J., Kent, R.L. and Ago, J.M. : Synergistic interactions between interleukin 1, tumor necrosis factor and lymphotoxin in bone resorption, *J. Immunol.*, 138 : 1464-1468, 1987.
  34. Stashenko, P., Dewhirst, F.E., Rooney, M.L., Desjardins, L.A. and Heeley, J.D. : Interleukin- $1\beta$  is a potent inhibitor of bone formation in vitro, *J. Bone Miner. Res.*, 2 : 559-565, 1987.
  35. Takahashi, N., Mundy, G.R. and Roodman, G.D. : Recombinant human Interferon- $\gamma$  inhibits formation of human osteoclast-like cells, *J. Immunol.*, 137 : 3544-3549, 1986.
  36. Tatakis, D.N. : Interleukin-1 and bone metabolism : A review, *J. Periodontol.*, 64 : 416-431, 1993.
  37. Tsujimoto, M., Yokota, S., Vilcek, J. and Weissmann, G. : Tumor necrosis factor provokes super-oxide anion generation from neutrophils, *Biochem. Biophys. Res. Commun.*, 137 : 1094-1100, 1986.
  38. Woo, J.-O., Kim, G.-S. and Cheong, D.-K. : Isolation and characterization of bone cell population, *J. Dent. College SNU*, 11 : 1-14, 1987.