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

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Effects of several cytokines(IL-1β, TNFα, and IFNγ) 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-1β, TNFα, and IFNγ, respectively or combined.

And acid phosphatase was determined by biochemical method. Alkaline phosphatase was assayed to determine the effects of IL-1β, TNFα, and IFNγ 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-1β and TNFα, whereas decreased by IFNγ. However, no significant changes in alkaline phosphatase activity was observed when the osteoblast-like cells were treated with IL-1β and TNFα. Interestingly, IFNγ showed stimulatory effect on alkaline phosphatase activity.

The number of calcified nodules was decreased by treatment of cultures with 1 ng/ml IL-1β, 20 ng/ml TNFα, and 500 u/ml IFNγ 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-1β, Tumor necrosis factor α, Interferon γ, Phosphatase, Bone remodeling

The objective of orthodontic treatment is to produce an occlusion which is healthy and functionally satisfactory, aesthetically satisfactory and stable[16]. 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[11,29] 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[5]. Neurotransmitters and cytokines play a regulatory role in orthodontic force-induced alveolar bone remodeling[5]. Dewhirst et al.[6] recently reported that in fetal rat long bones in vitro, IL-1 and PTH, which respctively 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 exibit receptors for parathyroid hormone, vitamin D metabolites, and prostaglandins indicate that osteoclast recruitment and activity involve cells of the osteoblast lineage[24]. Heath et al.[15] reported that osteoblasts responded to systemic and local resorbing agents by producing collagenase, and Chamber et al.[2,3] 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[22].

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-1β [12,19,21,27]. And also many cells produce TNFα and/or IFNγ and they affect on the variety of cells[1,7,10,17,18,30,31,32,33,37].

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-1β, tumor necrosis factor α, and interferon γ) 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-1β, TNFα, IFNγ) 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.[38]. 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, 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_2. 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 10^4 cells/well into 96-microwell culture plates and cultured in MEM. When the cells reached confluency, they were treated with IL-1β (0.1-1.0 ng/ml), TNFα(2-20 ng/ml) and IFNγ(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-1β + 20 ng/ml TNFα, 1 ng/ml IL-1β + 500 u/ml IFNγ, 20 ng/ml TNF α + 500 u/ml IFNγ, 1 ng/ml IL-1β + 20 ng/ml TNFα + 500 u/ml IFNγ. 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 μl of cell extract, 20 μl of 0.1 M sodium citrate buffer (pH 4.8), and 40 μl 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 μl 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 [20].

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

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

Data represent Mean±SEM of 6 replicates.
* Significantly different from control, $p<0.05$
** Significantly different from control, $p<0.01$

Table 2. Effect of TNFα on the acid and alkaline phosphatase activity in cultures of fetal rat osteoblast-like cells

Treatment (TNFα, ng/ml)	Phosphatase activity(μM PNP/h/mg protein)	
	Acid	Alkaline
0(Control)	0.78±0.06	1.25±0.11
2	0.72±0.10	1.35±0.13
10	1.05±0.15	1.30±0.08
20	1.36±0.12**	1.24±0.06

Data represent Mean±SEM of 6 replicates.
** Significantly different from control, $p<0.01$

Table 3. Effect of IFNγ on the acid and alkaline phosphatase activity in cultures of fetal rat osteoblast-like cells

Treatment (IFNγ, u/ml)	Phosphatase activity(μM PNP/h/mg protein)	
	Acid	Alkaline
0(Control)	0.78±0.06	1.25±0.11
50	0.85±0.10	1.32±0.17
250	0.51±0.05*	1.75±0.10*
500	0.41±0.06**	2.44±0.17**

Data represent Mean±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(μM PNP/h/mg protein)	
	Acid	Alkaline
0(Control)	0.85±0.07	1.33±0.10
IL-1β+TNFα	1.69±0.15**	1.42±0.02
IL-1β+IFNγ	1.27±0.15*	1.85±0.08**
TNFα+IFNγ	0.79±0.10	1.66±0.14
IL-1β+TNFα+IFNγ	1.01±0.09	1.80±0.15*

Data represent Mean±SEM of 6 replicates.
* Significantly different from control, $p<0.05$
** 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 x 10^4 cells/dish and maintained until confluency was reached. Cells were cultured in MEM supplemented with 50 μg/ml ascorbic acid and 10 mM β- glycerophosphate, and each cytokine(IL-1β, 1 ng/ml; TNFα, 20 ng/ml; IFNγ, 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.

(5) Statistical analysis

Data were analyzed using Student's t-test. Results are expressed as Means ± 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-1β and dosedependently, whereas alkaline phosphatase activity was not changed by any concentration of IL-1β(Table 1). 1 ng/ml IL-1β 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[a]
None(control)	100
IL-1β (1ng/ml)	13.3±2.6[b**]
TNF α (20ng/ml)	23.7±5.4[b**]
IFN γ (500U/ml)	60.2±8.4[b*]

[a] Values expressed as percent of control.
[b] mean ± 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±6
IL-1β (ng/ml)	0.01	101±4
	0.1	98±4
	1	101±8
TNF α (ng/ml)	0.2	96±4
	2	107±7
	20	103±6
IFN γ (U/ml)	5	103±5
	50	100±7
	500	97±7

Data are Mean±SEM

group. TNFα also increased acid phosphatase activity as dosedependent manner from 2 to 20 ng/ml of TNF α. However, no significant changes in alkaline phosphatase activity was observed when the osteoblast-like cells were treated with TNFα(Table 2). It is of the interest that IFNγ affected on both enzyme activities; acid and alkaline phosphatase. IFNγ 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-1β+TNFα and IL-1β+IFNγ were still higher than that of control, even though those did not showed synergistic effects. However, the stimulatory effect of TNFα 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±4
IL-1β (1ng/ml) + TNF α (20ng/ml)	94±7
IL-1β (1ng/ml) + IFN γ (500U/ml)	99±6
TNF α (20ng/ml) + IFN γ (500U/ml)	102±7
IL-1β + TNF α + IFN γ	95±6

Data are Mean±SEM

be abolished by IFNγ action. The increased activities of alkaline phosphatase induced by IFNγ generally were inhibited either by IL-1β or TNFα.

(2) Calcified nodule formation

The number of calcified nodules was decreased by treatment of cultures with 1 ng/ml IL-1β, 20 ng/ml TNFα, and 500 u/ml IFNγ 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-1β and TNFα was significantly different from control($p < 0.001$) and INFγ 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 β(-1 ng/ml), TNFα(-20 ng/ml), and IFNγ (-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-1β (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[36], IL-1β stimulates the synthesis of prostaglandins and thromboxane that have been

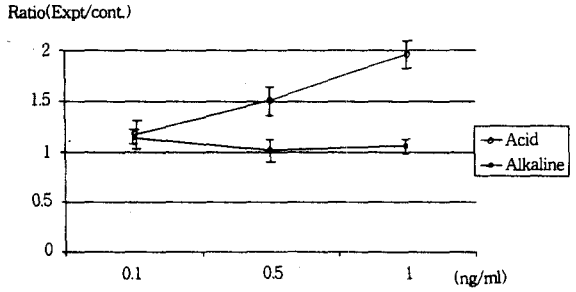

Fig 1. Effect of IL-1β on the acid and alkaline phosphatase activity in cultures of fetal rat osteoblast- like cells

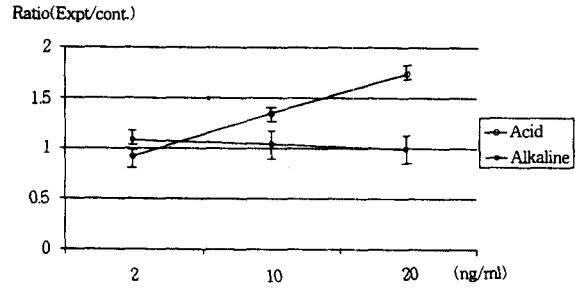

Fig 2. Effect of TNFα on the acid and alkaline phosphatase activity in cultures of fetal rat osteoblast- like cells

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

TNFα has been shown to induce the secretion of collagenase, prostaglandin E_2, and IL-6 by human fibroblasts and bone cells[4,8,23]. As shown in Figure 2, TNFα also stimulated acid phosphatase activity without a significant change of alkaline phosphatase. It has been reported similarly by Klebanoff et al.[18] that neutrophils are stimulated to release the lysosomal enzymes by TNFα. It suppressed the formation of calcified nodule when the osteoblast-like cells were treated with 20 ng/ml of TNFα for 21 days(Table 5). Taken together the results of IL-1β and TNFα, these two cytokines showed similar effects

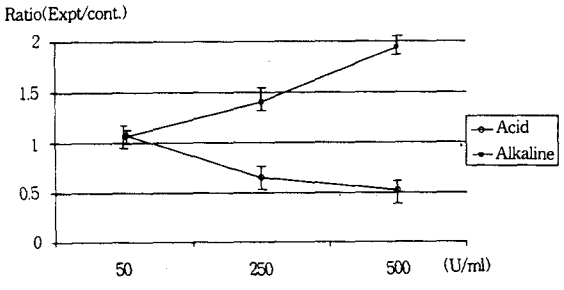

Fig. 3. Effect of IFNγ on the acid and alkaline phosphatase activity in cultures of fetal rat osteoblast- like cells

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-1β and TNFα 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. IFNγ has been shown to be a potent inhibitor of IL-1β, TNFα, parathyroid hormone, and vitamin D-stimulated bone resorption in vitro[9,13,28]. IFNγ also inhibits bone resorption by inhibition of recruitment and fusion of precursors for multinucleated or osteoclast-like cells in mouse and human marrow cultures[35]. In this study, it is of great interest that IFNγ 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-1β and TNFα. 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

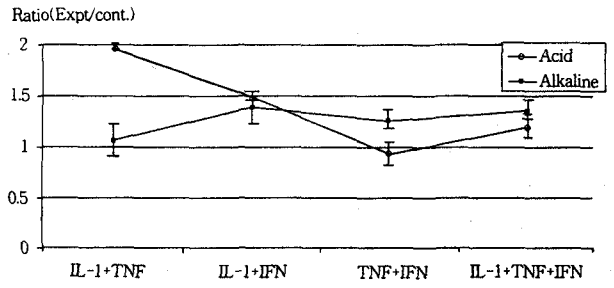

Fig. 4. Effect of cytokine combination on the acid and alkaline phosphatase activity in cultures of fetal rat osteoblast-like cells

when the cells were treated with IL-1β or TNFα. However, IFNγ did not affect on the calcified nodule size(Table 5).

In the experiment of cytokine combinations, IFNγ showed inhibitory effect on the acid phosphatase stimulated by IL-1β and TNFα (Figure 4). This inhibitory effects of IFNγ on the IL-1β and TNFα can be in accordance with the reports concerning to the osteoclastic bone resorption induced by several cytokines and osteotropic hormones[9,14,31].

It can be concluded from the present study that IL-1β and TNFα increase the acid phosphatase activity and inhibit the mineralization without any influence on alkaline phosphatase activity. Whereas IFNγ 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 osteoblasic activity. And acid and alkaline phosphatase was assayed to determine the effects of IL-1β, TNFα, and IFNγ 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 increased by the addition of IL-1β and TNFα, whereas decreased by IFNγ.
2. No significant changes in alkaline phosphatase activity was observed when the osteoblast-like cells were treated with IL-1β and TNFα.
3. IFNγ showed stimulatory effect on alkaline phosphatase activity.
4. The number of calcified nodules was decreased by treatment of cultures with 1 ng/ml IL-1β, 20 ng/ml TNFα, and 500 u/ml IFNγ.

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