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# Effect of Cytokines and bFGF on the Osteoclast Differentiation Induced by 1 $\alpha$,25-$(OH)_2D_3$ in Primary Murine Bone Marrow Cultures

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Bone is a complex tissue in which resorption and formation continue throughout life. The bone tissue contains various types of cells, of which the bone forming osteoblasts and bone resorbing osteoclasts are mainly responsible for bone remodeling. Periodontal disease represents example of abnormal bone remodeling. Osteoclasts are multinucleated cells present only in bone. It is believed that osteoclast progenitors are hematopoietic origin, and they are recruited from hematopoietic tissues such as bone marrow and circulating blood to bone. Cells present in the osteoclast microenvironment include marrow stromal cells, osteoblasts, macrophages, T-lymphocytes, and marrow cells. These cells produce cytokines that can affect osteoclast formation. In vitro model systems using bone marrow cultures have demonstrated that IL-1 $\beta$, IL-3, TNF- $\alpha$, bFGF can stimulate the formation of osteoclasts. In contrast, IL-4 inhibits osteoclast formation. Knowledge of cytokines and bFGF that affect osteoclast formation and their capacity to modulate the bone-resorbing process should provide critical insights into normal calcium homeostasis and disorders of bone turnover such as periodontal disease, osteoporosis and Paget's disease.

Key Words: Cytokines, Basic fibroblast growth factor, Bone marrow, Osteoclast formation

## INTRODUCTION

Bone is an important target tissue for immune cell products and may be affected by these products during both pathologic and physiologic conditions in vivo. The osteoclast is the cell that resorbs bone. Osteoclastic bone resorption is a complex process that involves the release of mineral from bone and then degradation of the proteinaceous bone matrix (Mundy, 1993). How osteoclasts are formed and activated or the mechanisms by which bone is resorbed by osteoclasts remain unclear. Osteoclasts are derived from hemopoietic precursors, from a lineage shared with mononuclear phagocytes, and immature precursor cells migrate to bone surfaces from an extraosseous source (Fuller et al, 1998).

Interleukin (IL-1) is produced by monocytes and marrow stromal cells and can stimulate bone resorption in organ culture (Sugawara et al, 1998). Interleukin-3 (IL-3) is produced by T lymphocytes and other cell types and has a hematopoietic differentiating capability (Sarma & Flanagan, 1996). Interleukin-4 (IL-4) is first identified as a lymphocyte growth factor, but it has become increasingly evident that it is a functional regulator for a variety of cells, including the bone-forming cells osteoblasts and hemopoietic cells (Ueno et al, 1992; Okada et al, 1998).

1 $\alpha$,25-$(OH)_2D_3$, an active form of osteotropic

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hormone vitamin $D_3$, has been reported to stimulate bone resorption and induce the differentiation of mouse myeloid leukaemia cells into macrophage-like cells. It directly stimulates the fusion of mouse alveolar macrophages to form multinucleated giant cells (Akatsu et al, 1989).

Tumor necrosis factor-$\alpha$ (TNF-$\alpha$) has been proposed as a local mediator of the control of bone turnover in situations of chronic inflammation, and it has been assumed that the local source of TNF-$\alpha$ is the monocyte in the adjacent bone marrow or the local circulation (Assuma et al, 1998).

Basic fibroblast growth factor (bFGF) is a potent stimulator of osteoblast replication (Globus et al, 1988). It is produced by bone cells (Globus et al, 1989) and stored in the extracellular matrix (Hauschka et al, 1986). Basic fibroblast growth factor increases bone resorption in fetal rat long bone and mouse calvariae cultures (Kawaguchi et al, 1995).

In this study we have examined the effects of recombinant mouse IL-1$\beta$, IL-3, IL-4, and TNF-$\alpha$, cytokines that have potent effects on bone resorption, on the formation of multinucleated cells (MNC) in mouse bone marrow cultures. We demonstrate that these cytokines are potent stimulators of osteoclast-like cell formation.

## METHODS

### *Animals*

The original stock of ICR mice was purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and maintained in the Animal Facility Units of Wonkwang University School of Dentistry (Iksan, Korea). Mice of 6 to 7 weeks of age were used to obtain bone-marrow cells.

### *Reagents*

Recombinant mouse interleukin-1$\beta$ (25 $\mu$g/ml), recombinant mouse interleukin-3 (100 $\mu$g/ml), recombinant mouse interleukin-4 (1.5 $\mu$g/ml), recombinant mouse tumor necrosis factor-$\alpha$ (10 $\mu$g/ml) were obtained from Genzyme (Mnchen, Germany). Basic fibroblast growth factor and 1$\alpha$,25-$(OH)_2D_3$ were purchased from Calbiochem (La Jolla, CA, USA). All culture wares were purchased from Nunc Inc. (North Aurora Road, IL, USA). $\alpha$-Minimum essential medium, Hank's balanced salt solution, fetal bovine serum and other tissue culture reagents were bought from GIBCO Co. (Gaithersburg, MD, USA).

### *Bone marrow culture*

Mice were sacrified by cervical dislocation under light ether anaesthesia and tibiae of the mice were aseptically removed (Chae et al, 1998). Both ends of the bone were cut off and marrow cavity was flushed with 1 ml $\alpha$-minimum essential medium. The marrow cells were collected, washed twice with $\alpha$-minimum essential medium and maintained in $\alpha$-minimum essential medium containing 10% fetal bovine serum at $3\times10^6$ cells/ml in 24-well plates. Various concentrations of interleukin-1$\beta$, interleukin-3, interleukin-4, tumor necrosis factor-$\alpha$, basic fibroblast growth factor and 1$\alpha$,25-$(OH)_2D_3$ were added at the beginning of culture as well as at each time of medium change every two days. All cultures were maintained at 37°C in a humidified atmosphere of 5% $CO_2$ in air.

### *Enzyme histochemistry*

After culture for indicated periods, adherent cells were fixed with 10% formalin in phosphate buffered saline for 10 min. And then fixed with ethanol-acetone (50 : 50, vol/vol) for 1 min. The cells were dried and stained for tartrate-resistant acid phosphatase in 0.1 M sodium acetate buffer (pH 5.0), containing AS-MX phosphate and red violet LB salt in 50 mM sodium tartrate. Tartrate-resistant acid phosphatase-positive cells containing three or more nuclei were counted as osteoclast-like multinucleated cells (Burstone, 1958).

### *Statistical analysis*

Data were represented as the mean$\pm$S.E.M. of four experiments. Each of experiment was quadruplicated. The statistical significance of the difference was determined by paired Student's t-test.

## RESULTS

Mouse bone marrow cultures were treated with 1$\alpha$,25-$(OH)_2D_3$ to generate tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs).

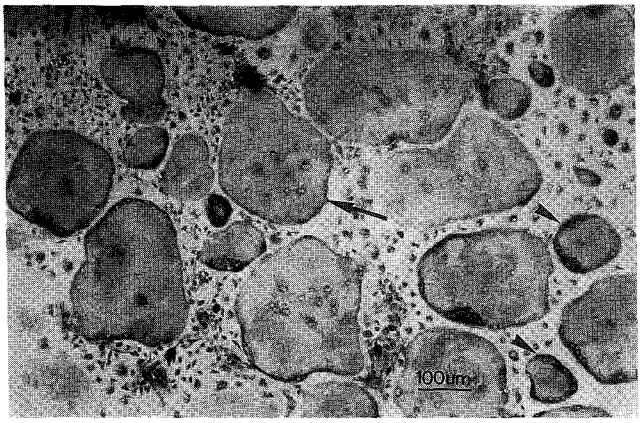


**Fig. 1.** Tartrate-resistant acid phosphatase-positive multinucleated cells formed in cultures of mouse bone marrow cells (×100). Mouse bone marrow mononuclear cells ($3 \times 10^6$ cells/well) were treated with $10^{-7}$ M of 1 $\alpha$,25-$(OH)_2D_3$ for 8 days. Cells were then fixed and stained for tartrate-resistant acid phosphatase. Nuclei located in the centre (arrows) and in the periphery of the cells (arrowhead) were shown in tartrate-resistant acid phosphatase-positive multinucleated cells.

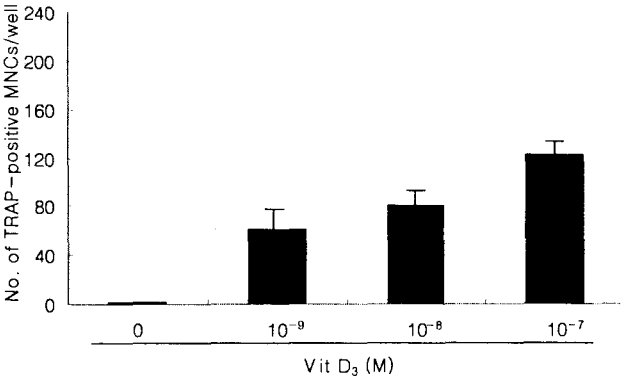


**Fig. 2.** Effect of 1 $\alpha$,25-$(OH)_2D_3$ on the generation of tartrate-resistant acid phosphatase-positive multinucleated cells. Mouse bone marrow mononuclear cells ($3 \times 10^6$ cells/well) were cultured for 8 days with vehicle, ethanol, or 1 $\alpha$,25-$(OH)_2D_3$. Data were expressed as the mean ± S.E.M. of four cultures. TRAP=tartrate-resistant acid phosphatase. MNC=Multinucleated cells.

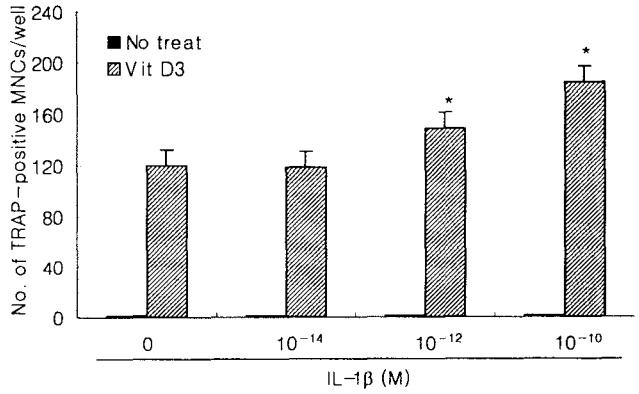


**Fig. 3.** Effect of interleukin-1 $\beta$ (IL-1 $\beta$) on the generation of tartrate-resistant acid phosphatase-positive multinucleated cell in cultures of mouse bone marrow cells. Mouse bone marrow mononuclear cells ($3 \times 10^6$ cells/well) were cultured 8 days with or without 1 $\alpha$,25-$(OH)_2D_3$. After adding of various concentrations of IL-1 $\beta$, the cells were stained and counted the number of tartrate-resistant acid phosphatase-positive multinucleated cells. The results were expressed as the mean ± S.E.M. of four cultures. *$p < 0.05$, compared with 1 $\alpha$,25-$(OH)_2D_3$ alone.

Addition of 1 $\alpha$,25-$(OH)_2D_3$ ($10^{-7}$ M) induced the formation of tartrate-resistant acid phosphatase-positive multinucleated cells in mouse marrow cultures for 8 days. At least two types of tartrate-resistant acid phosphatase-positive multinucleated cells were detected: One in which nuclei were located in the center of the cells and the other in which nuclei were located in the periphery of the cells (Fig. 1). 1 $\alpha$,25-$(OH)_2D_3$ induced the generation of tartrate-resistant acid phosphatase-positive multinucleated cells in a dose-dependent manner (Fig. 2). Cells without 1 $\alpha$,25-$(OH)_2D_3$ showed very few TRAP multinucleated cells.

Interleukin-1 $\beta$ (IL-1 $\beta$) promotes bone resorption in vitro (Lorenzo et al, 1987) and in vivo (Boyce et al, 1989). We tested whether interleukin-1 $\beta$ stimulates the formation of osteoclast-like multinucleated cells in mouse bone marrow cultures, based on assuming tartrate-resistant acid phosphatase-positive multinucleated cells as osteoclasts (Chae et al, 1998). Mouse bone marrow culture was done with IL-1 $\beta$ and 1 $\alpha$,25-$(OH)_2D_3$ ($10^{-7}$ M) for 8 days. Interleukin-1 $\beta$ significantly increased tartrate-resistant acid phosphatase-positive multinucleated cells at $10^{-12} \sim 10^{-10}$ M (Fig. 3). The murine cytokine interleukin-3 (IL-3), also known as multipotential colony-stimulating factor (Cutler et al, 1985) and persistent cell-stimulating factor (Clark-Lewis et al, 1984), is distinct among the cloned hematopoietic-stimulating factors in having the capacity to stimulate progenitor cells renewal. We examined the effect of interleukin-3 on the formation of osteoclast in mouse bone marrow cultures. Interleukin-3 significantly increased tartrate-resistant acid phosphatase-positive multinucleated cells

at 100~1000 U/ml (Fig. 4).

The proinflammatory cytokine, tumor necrosis factor-$\alpha$, is known to enhance bone resorption in murine cultures and in humans and mice in vivo (Mundy, 1995). We evaluated the effect of tumor necrosis factor-$\alpha$ on the generation of osteoclast. Tumor necrosis factor-$\alpha$ significantly increased tartrate-resistant acid phosphatase-positive multinucleated cells at $10^{-10}$~$10^{-8}$ M (Fig. 6). These results demonstrated that interleukin-1$\beta$, interleukin-3, interleukin-6, and tumor necrosis factor-$\alpha$ could dose-dependently and significantly augment the generation of

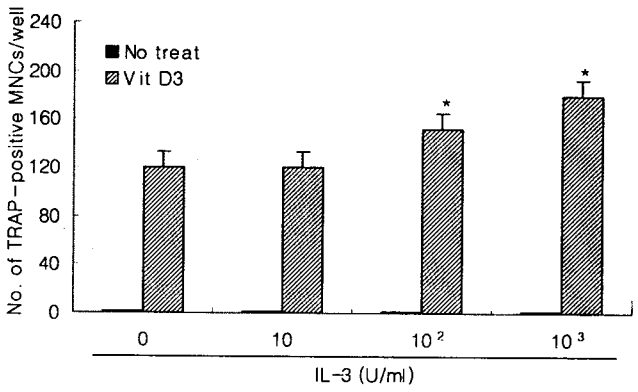


**Fig. 4.** Effect of interleukin-3 (IL-3) on the generation of tartrate-resistant acid phosphatase-positive multinucleated cells in mouse bone marrow cultures. Mouse bone marrow mononuclear cells ($3\times10^6$ cells/well) were cultured for 8 days with or without $10^{-7}$ M 1 $\alpha$,25-$(OH)_2D_3$. Various concentrations of IL-3 were added on the cells. The results were expressed as the mean±S.E.M. of four cultures. *$p<0.05$, compared with 1 $\alpha$,25-$(OH)_2D_3$ alone.

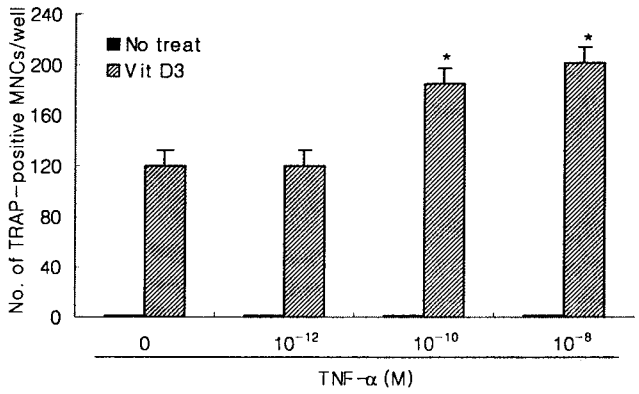


**Fig. 6.** Effect of tumor necrosis factor-$\alpha$ (TNF-$\alpha$) on the generation of tartrate-resistant acid phosphatase-positive multinucleated cells. Mouse bone marrow mononuclear cells ($3\times10^6$ cells/well) were cultured for 8 days with or without $10^{-7}$ M 1 $\alpha$,25-$(OH)_2D_3$. Various concentrations of TNF-$\alpha$ were added on the cells. The results were expressed as the mean±S.E.M. of four cultures. *$p<0.05$, compared with 1 $\alpha$,25-$(OH)_2D_3$ alone.

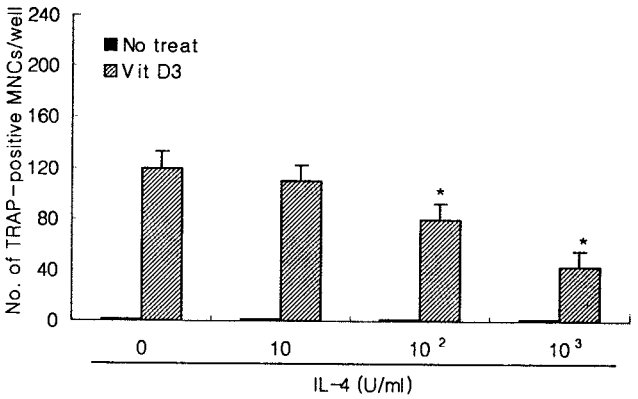


**Fig. 5.** Effect of interleukin-4 (IL-4) on the generation of tartrate-resistant acid phosphatase-positive multinucleated cells in mouse bone marrow cultures. Mouse bone marrow mononuclear cells ($3\times10^6$ cells/well) were cultured for 8 days with or without $10^{-7}$ M 1 $\alpha$,25-$(OH)_2D_3$. Various concentrations of IL-4 were added on these cells. The results were expressed as the mean± S.E.M. of four cultures. *$p<0.05$, compared with 1 $\alpha$,25-$(OH)_2D_3$ alone.

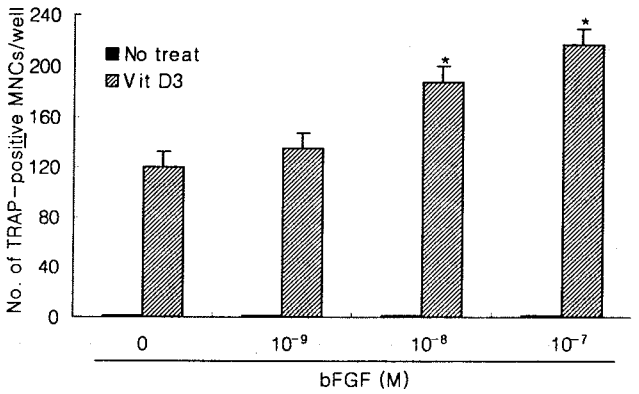


**Fig. 7.** Effect of basic fibroblast growth factor (bFGF) on the generation of tartrate-resistant acid phosphatase-positive multinucleated cells. Mouse bone marrow mononuclear cells ($3\times10^6$ cells/well) were cultured for 8 days with or without $10^{-7}$ M 1 $\alpha$,25-$(OH)_2D_3$. Various concentrations of bFGF were added on the cells. The results were expressed as the mean±S.E.M. of four cultures. *$p<0.05$, compared with 1 $\alpha$,25-$(OH)_2D_3$ alone.

osteoclast induced by 1 $\alpha$,25-$(OH)_2D_3$.

Interleukin-4 inhibited bone resorption stimulated by systemic and local bone-resorbing agents in vitro (Watanabe et al, 1990) and in vivo (Nakano et al, 1994). We determined the effect of interleukin-4 on the formation of osteoclast from mouse bone marrow culture. Mouse bone marrow cell culture was done in interleukin-4 and 1 $\alpha$,25-$(OH)_2D_3$ ($10^{-7}$ M) for 8 days. Interleukin-4 significantly inhibited tartrate- resistant acid phosphatase-positive multinucleated cells at 1 ~ 10 U/ml (Fig. 5).

Basic fibroblast growth factor is a potent mitogen for stromal cells (Oliver et al, 1990) and these cells also produce this growth factor. Gabrilove et al recently demonstrated that bFGF increases the formation of CFU-GM (colony-forming unit for granulocytes and macrophages) in human marrow cultures.

We examined the effect of bFGF on the formation of osteoclast in mouse bone marrow cultures. bFGF significantly increased tartrate-resistant acid phosphatase-positive multinucleated cells at 1 ~ 100 nM (Fig. 7).

## DISCUSSION

Osteoclasts are multinucleated giant cells that mediate the process of bone resorption (Mundy, 1993). Osteoclasts do not replicate by cell division (Scheven et al, 1985). Instead, they increase their size and number through the fusion and incorporation of a terminally differentiated cell, the osteoclast precursor.

These multinucleated giant cells form by fusion of mononuclear precursors derived from hemopoietic progenitor cells (Mundy, 1987; Fuller et al, 1998). We have developed (Chae et al, 1998) a long-term mouse bone marrow culture system in which multinucleated cells (MNC) form. Formation of multinucleated cells in mouse bone marrow is appropriately regulated by osteotropic hormones and factors. PTH, 1 $\alpha$,25-$(OH)_2D_3$, TNF- $\alpha$, IL-1 $\beta$, and IL-6, which stimulate osteoclastic bone resorption in vitro, increased the number of multinucleated cells in mouse bone marrow cultures.

Interleukin-1 (IL-1) was the first cytokine identified for its effects on osteoclastic bone resorption. It is a very powerful bone resorption stimulator (Sugawara et al, 1998). The effects of IL-1 on cells in the osteoclast lineage have not been completely worked out. It stimulates bone resorption in all organ culture systems, including fetal rat long bones and neonatal mouse calvariae (Gowen & Mundy, 1986). It enhances the formation of cells with osteoclast characteristics in cultures of human marrow cells (Pfeilschifter et al, 1989). It also stimulates the capacity of isolated osteoclasts to form resorption lacunae on calcified matrices (Thomson et al, 1986). This evidence has been taken to suggest that IL-1 has effects only indirectly on osteoclastic bone resorption. The present study demonstrates that mouse recombinant IL-1 $\beta$ markedly stimulated the formation of osteoclast-like MNC in mouse bone marrow cultures. These results suggest that the formation of new osteoclasts is an important mechanism by which IL-1 $\beta$ stimulates osteoclastic bone resorption.

Interleukin-3 (IL-3), a cytokine produced by T lymphocytes and other cell types, can both increase the number of bone marrow cells that become TRAP-positive, and also induce fusion of TRAP-positive cells into polykaryons. Such cells morphologically resemble osteclasts produced in bone marrow cultures by treatment with 1 $\alpha$,25-$(OH)_2D_3$. That IL-3, known to have broad hematopoietic effects (Sarma & Flanagan, 1996) can also influence the cell type involved in bone resorption has further implications for the influence of the immune system on the skeletal system in various disorders, such as rheumatoid arthritis.

Interleukin-4 (IL-4) is a 20-kd immunoregulatory glycoprotein that is produced and secreted by activated T lymphocytes and mast cells. This cytokine functions as a growth and/or differentiation factor for a wide variety of cells of hematopoietic lineage (Kupper et al, 1987; Rennick et al, 1987; Peschel et al, 1989; Abramson & Gallin, 1990; Okada et al, 1998). In addition to these effects, IL-4 has been shown to influence skeletal metabolism (Watanabe et al, 1990). In this study, IL-4 inhibited the in vitro bone resorption induced by a wide variety of agents such as PTH, 1 $\alpha$,25-$(OH)_2D_3$, IL-1 $\beta$ and $PGE_2$, which in excess have been implicated in both osteoporosis and hypercalcemia of malignancy (Mundy et al, 1984). Recently, it has been reported that IL-4 induced in vitro mineralization in human osteoblast-like cells (Ueno et al, 1992). Although these observations established IL-4 as a potent antiosteolytic factor, the mechanism underlying its effects remained poorly characterized. Because IL-4 inhibited the resorption-inducing effects of such a diverse group of osteolytic substances, we reasoned that this cytokine impacted essential resorptive processes. In this study,

we found that IL-4 significantly antagonized osteoclast generation in vitro in an in vitro hematopoietic model of osteoclastic cell formation using mouse bone marrow cell.

Tumor necrosis factor-$\alpha$ (TNF-$\alpha$) is a pleiotropic hormone with actions on the differentiation, growth, and functional activities of normal and malignant cells from numerous tissues (Beutler & Cerami, 1986; Maury, 1986). Its actions on connective tissue cells include the stimulation of bone resorption, cartilage breakdown (Saklatvala, 1986; Assuma et al, 1998), collagenase production by synovial cells (Dayer et al, 1985), and the inhibition of rat calvarial bone collagen synthesis (Bertolini et al, 1986; Smith et al, 1987). TNF-$\alpha$ has been proposed as a local mediator of the control of bone turnover in situations of chronic inflammation, and it has been assumed that the local source of TNF-$\alpha$ is the monocyte in the adjacent bone marrow or the local circulation. TNF-$\alpha$ is a potent inducer of bone resorption in fetal rat and mouse organ cultures (Bertolini et al, 1986). The present study demonstrated that TNF-$\alpha$ enhanced the generation of osteoclast-like cells in mouse bone marrow cultures. This result is consistent with previous reports that TNF-$\alpha$ stimulated osteoclast-like cell formation in long-term human marrow cultures (Pfeilschifter et al, 1989). TNF-$\alpha$ stimulates both proliferation and differentiation of precursors for osteoclast-like cells to osteoclasts.

bFGF is produced locally in bone by osteoblastic cells and may be deposited in bone matrix. It may be released from matrix by osteoclastic activity when bone resorption is initiated and have effects on local bone turnover. Alternatively, it may be released from bone at the site of a recent fracture when the matrix is disrupted (Globus et al, 1989). Because of its ability to initiate a osteoclast-like cell formation, bFGF could be involved in the recruitment and activation of osteoclast-like precursors.

These results demonstrate that interleukin-1$\beta$, interleukin-3 interleukin-4, basic fibroblast growth factor, and tumor necrosis factor-$\alpha$ are the important members of cytokine network that control osteoclast development. The degree to which the cell is actually committed to the osteoclast pathway appears to be influenced by soluble mediators produced by cells of the immune system. These results may greatly enhance our understanding of the role that cytokines play in the regulation of normal and pathologic bone resorption. More extensive studies will be required to determine the precise role of IL-1$\beta$, IL-3, IL-4, and TNF-$\alpha$ in the osteoclastogenic effects at the molecular level.

## ACKNOWLEDGEMENTS

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