# Effects of Bone Resorbing Agents on the Expression of IL−6 in Osteoblastic Cells*

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학술

## ABSTRACT

**수종 골흡수촉진물질들이 조골세포의 IL−6 발현에 미치는 영향**

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Interleukin−6 (IL−6)는 여러 세포와 조직에서 다양한 기능을 조절하는 multifunctional cytokine으로, 조골세포에서도 합성 분비되며 생리적 또는 병적 상황에 따라 골흡수의 중요한 조절인자로 작용하는 것으로 알려져 있다. 한편 골흡수를 촉진하는 것으로 알려진 많은 물질들은 − parathyroid hormone (PTH), 1,25−dihydroxyvitamin $D_3$ (1,25−$(OH)_2D_3$)와 같은 전신적 홀몬이나 interleukin−1 (IL−1), tumor necrosis factor−$\alpha$ (TNF−$\alpha$)와 같은 국소조절인자 등 − 조골세포를 통하여 작용하는 것으로 알려져 있으므로, 이들 골흡수물질들이 조골세포에서의 IL−6의 생성을 조절함으로써 그 효과를 나타낼 가능성이 있을 것으로 생각되어 진다. 그러므로 본 실험에서는 각각 사람과 백서의 골종양에서 유래한 조골세포유사세포인 MG−63과 ROS 17/2.8 세포를 이용하여 PTH, 1,25$(OH)_2D_3$, IL−1$\beta$ 및 TNF−$\alpha$가 이들 세포에서의 IL−6 발현에 미치는 영향을 관찰함으로써 IL−6가 이들 골흡수촉진물질들의 매개인자로 작용할 가능성의 여부를 알아보고자 하였다. MG−63과 ROS 17/2.8 세포 모두 약물로 처리하지 않은 대조군에서는 IL−6의 발현이 거의 관찰되지 않았다. 한편 MG−63세포에서는 IL−1$\beta$와 TNF−$\alpha$ 에 의하여 IL−6의 발현이 증가되었으나, PTH와 1,25−$(OH)_2D_3$에 의해서는 조절되지 않았다. 시간에 따른 IL−6의 발현 조절 양상을 관찰하기 위하여 MG−63 세포에 IL−1$\beta$ (1.0 ng/ml)와 TNF−$\alpha$ (20 ng/ml)를 첨가하고 6, 12, 24, 또는 48시간 후에 관찰하였을 때, 6시간후에 미량의 IL−6 mRNA가 관찰되었으며 그 후 48시간까지 그 양이 점차 증가되었다. 또한 MG−63세포에서 IL−1$\beta$ 는 0.1 − 10 ng/ml의 범위에서 농도의존적으로 IL−6의 발현을 증가시켰으며, 그 효과는 TNF−$\alpha$ 보다 더 크게 나타남이 관찰되었다. 한편 ROS 17/2.8 세포에서는 MG−63세포에서와는 달리 어떤 약물에 의해서도 IL−6 발현 조절이 관찰되지 않았다. 이상의 실험결과는 IL−1$\beta$ 와 TNF−$\alpha$ 와 같은 cytokine들의 조골세포 IL−6 생성 촉진효과가 이들의 골흡수 촉진 작용을 부분적으로는 매개할 수 있음을 시사하는 것으로 여겨지며, 이러한 IL−6의 생성촉진은 조골세포의 IL−6 mRNA의 증가에 의한 것으로 생각된다.

**주요어** ; 조골세포, IL−6, IL−1$\beta$, TNF−$\alpha$

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## I. Introduction

Interleukin−6 (IL−6), 21−28 kD group of modified phosphoglycoproteins, is a multifunctional cytokine that regulates pleiotropic functions of cells and tissues. IL−6 is produced by various cell types, including monocytes, fibroblasts, keratinocytes, endothelial cells, and osteoblasts, and is a potent paracrine factor for hematopoiesis, B cell growth and differentiation, immune responses, and acute−phase reactions[1,2].

Recently several lines of evidence have suggested that IL−6 also plays a role as an osteotropic agent in diseases associated with abnormal bone loss. Fukumoto *et al.*[3] and Ohsaki *et al.*[4] demonstrated that excessive bone resorption seen in patients who pertain pheochromocytoma or giant cell tumors of bone is attributable to IL−6 secreted by tumor cells. Roodman *et al*.[5] found that conditioned media from pagetic marrow cultures stimulated osteoclast formation in normal marrow cultures and this stimulatory effect is, at least in part, ascribed to IL−6 secreted in an autocrine/paracrine fashion. IL−6 has been also implicated in the pathophysiology of rheumatoid arthritis. High levels of IL−6 was detected in synovial fluid from patients with inflammatory arthropathies associated with local bone resorption and cells obtained from synovial fluid constitutively express IL−6 mRNA[6,7]. And the role of IL−6 has been demonstrated in osteoporosis associated with estrogen deficiency[8,9] and alveolar bone destruction in periodontal disease as well[10].

In addition to these possible roles of IL−6 in bone resorption, other groups have reported that IL−6 was produced by bone rudiments and osteoblasts. Feyen *et al.*[11] and Al−Humidan *et al.*[12] reported that IL−6 was released from mouse bones in organ culture. Lowik *et al.*[13] and Ishimi *et al.*[14] reported that IL−6 was secreted by rat and mouse osteoblastic cells, respectively. The regulation of expression and secretion of IL−6 in bone by osteotropic hormone and/or local factors has been reported. Feyen *et al.*[11] and Greenfield *et al.*[15] demonstrated that parathyroid hormone (PTH) stimulated mRNA expression and secretion of IL−6 in murine osteoblastic cells. And Al−Humidan *et al.*[12] reported that interleukin−1$\alpha$ (IL−1$\alpha$), PTH, and 1,25−dihydroxyvitamin $D_3$ (1,25$(OH)_2D_3$) induced IL−6 release by mouse calvaria. Littlewood *et al.*[16] also showed that human osteoblasts produced IL−6 in response to several external stimuli such as IL−1 and lipopolysaccharide, suggesting that IL−6 produced by osteoblasts has possible paracrine effects on bone resorption or bone formation. However, contrary to above reports, works by Littlewood *et al.*[16] and Linkhart *et al.*[17] showed that PTH and 1,25$(OH)_2D_3$ had no effect on IL−6 secretion in human osteoblastic cells.

Taken together, these reports suggest that IL−6 plays an important role in stimulating bone resorption in some physiologic and/or pathologic conditions and may mediate, at least partly, the action of certain systemic or local bone resorptive agents. However, the role of IL−6 as a possible mediator of bone resorptive action of systemic or local osteotropic agents is not clear yet. Therefore, we observed the regulation of IL−6 mRNA expression in human and rat osteoblastic cells by several systemic osteotropic hormones and cytokines, known to stimulate bone resorption.

## II. Materials and Methods

Materials : Media, fetal bovine serum (FBS), and other cultural reagents were obtained from GIBCO BRL (Grand Island, NY, USA) and plastic culture wares from Corning Inc. (Corning, NY, USA). 1,25$(OH)_2D_3$ was obtained from Calbiochem−Novobiochem Corp. (San Diego, CA, USA) and recombinant human interleukin−1$\beta$ (IL−1$\beta$) and tumor necrosis factor−$\alpha$ (TNF−$\alpha$) from Genzyme (Cambridge, MA, USA). Synthetic PTH (1−84) was kindly provided by Dr. Park at Mogam Biotechnology Research Institute (Yongin−Kun, Kyonggi−Do, Korea). $^{32}$P−dCTP, megaprime™ DNA labelling system, and Hybond−N nylon membrane were purchased from Amersham Life Science (Arlington Heights, IL, USA). RNeasy™ RNA minipreparation kit was from Qiagen (Chatsworth, CA, USA) and Bio−Spin chromatography column from BIO−RAD (Hercules, CA, USA). Molecular biology−grade reagents for northern blot analysis and X−OMAT AR films were from IBI / Eastman Kodak (New Haven, CT, USA) and Sigma (St. Louis, MO, USA). A human IL−6 cDNA probe was purchased from American Type Culture Collection (ATCC, Rockille, MD, USA).

Osteoblastic Cell Cultures : MG−63, human osteoblastic cells derived from human osteosarcoma, was purchased from ATCC and maintained with Dulbecco's modified Eagle media (DMEM) containing 10% FBS. ROS 17/2.8, rat osteoblastic cells derived from rat osteosarcoma, was kindly provided by Dr. Hauschka at Harvard School of Dental Medicine (Boston, MA, USA) and maintained in Ham's F12 medium containing 5 % FBS. To test the effect of various agents on IL−6 expression, the cells were grown to about 70% confluence in 100 mm tissue culture dishes and then incubated in serum−free medium for 24 hrs. PTH ($10^{-8}$ M), 1,25$(OH)_2D_3$ ($10^{-7}$ M), IL−1$\beta$ (1 ng/ml), or TNF−$\alpha$ (20 ng/ml) was added and cells were incubated for 24 hrs before total RNAs isolation. The time course of IL-6 induction was determined by treating cells with inducible agents for 6, 12, 24, and 48 hrs and measuring IL-6 mRNA levels in northern blots.

Isolation of Total Cellular RNAs and Northern Blot Analysis : Total cellular RNAs were isolated using RNeasy™ RNA isolation kit. Twenty micrograms of each RNAs were separated on a 1.2 % (wt/vol) formaldehyde-denaturing agarose gel and transferred to a nylon membrane (Hybond-N) for northern blot analysis. The RNA blots were hybridized for 12 h at 42℃ with a complementary DNA to human IL-6 labeled with $^{32}P$-dCTP by random primer extension (megaprime DNA labelling kit) and purified using Bio-Spin chromatography column. Hybridizing solution contains 50% formaldehyde / 10% dextran sulfate / 5X SSPE (0.15 M NaCl, 0.01 M $Na_2HPO_4$, 0.01 M EDTA) / 2.5X Denhardt's solution / 100 $\mu$g/ml denatured salmon sperm DNA. The blots were washed twice with 5X SSPE for 15 min at 42℃, and analyzed by autoradiography. RNA loading equivalance was ensured by comparing the transcript band intensity to the corresponding 18S rRNA density in autoradiogram.

## III. Results

The steady state levels of IL-6 mRNA in MG-63 cells treated with various bone-resorbing agents are shown in Fig. 1. After being cultured in serum-free medium, MG-63 cells were treated with various systemic and local bone resorbing agents for 24 hrs. The transcript of IL-6 was detected as a single 1.3 kb band. The level of IL-6 mRNA was extremely low in the untreated MG-63 cells. Neither PTH nor $1,25(OH)_2D_3$ induced IL-6 mRNA expression in these cells. On the other hand, treatment with 20 ng/ml of TNF-$\alpha$ and 1 ng/ml of IL-1$\beta$ greatly increased the expression of IL-6 mRNA. The potency of TNF-$\alpha$ was lower than that of IL-1$\beta$.

Fig. 2 and 3 show a time course of change in the steady state levels of IL-6 mRNA in MG-63 cells cultured with IL-1$\beta$ (Fig. 2) or TNF-$\alpha$ (Fig. 3). After being cultured in serum-free medium, the cells were treated for the indicated time periods with 1 ng/ml of IL-1$\beta$ or 20 ng/ml of TNF-$\alpha$. The expression of IL-6 mRNA increased slightly at 6 hrs after adding IL-1$\beta$ or TNF-$\alpha$ and increased progressively up to 48 hrs.

The dose-response effects of IL-1$\beta$ and TNF-$\alpha$ in inducing IL-6 mRNA expression in MG-63 cells are shown in Fig. 4 and 5. After being cultured in serum-free medium, the cells were treated for 24 hrs with graded concentrations of IL-1$\beta$ or TNF-$\alpha$. The expression of IL-6 mRNA was dose-dependently increased by IL-1$\beta$ (Fig. 4). In TNF-$\alpha$ treated cells, the maximum expression of IL-6 mRNA was induced by 10 ng/ml of TNF-$\alpha$ and no more induction was observed in cells treated with higher concentration of TNF-$\alpha$.

As in MG-63 cells, the basal expression of IL-6 mRNA was barely detectable in ROS 17/2.8 cells. Dissimilar to the results in MG-63 cells, however, the marked stimulation of IL-6 mRNA expression did not occurred in ROS 17/2.8 cells by any treatment with above bone resorbing agents when observed at 24 hrs after addition (data not shown).

## IV. Discussion

Bone is a dynamic structure which is constantly remodeled throughout the life. During times of homeostasis, bone mass is maintained via the coupling of bone formation and bone resorption[18,19]. Classic studies have shown that osteoblasts are the major mediators of bone formation, osteoclasts are the the major mediators of bone resorption, and systemic hormones such as PTH are important regulators of the balance between the activities of these cells[18,19]. However, many reports over the last several years showed the complexity of the cell–cell interactions involved in these processes and, in addition to systemic hormones, inflammatory cytokines like IL–1 and TNF are also important regulators of osteoblast and osteoclast activities[18,19]. And it is also known that in addition to producing new bone, osteoblasts control the bone resorption process by elaborating soluble factors that act to regulate the formation and activation of osteoclasts and that the effects of several systemic hormones such as PTH or $1,25(OH)_2D_3$ and local regulators such as IL–1 or TNF on bone resorption are at least partly mediated by osteoblast–derived soluble factors[20–25]. However the nature of these important soluble factors is incompletely understood.

Recently much attention has focused on the cytokine IL–6 as an important regulator of bone cell function[5]. The majority of *in vitro* evidence suggests that IL–6 is an osteotropic factor whose predominant effects are on cells of the osteoclast lineage[4,5,13,14,26]. Lowik *et al.*[13] and Ishimi *et al.*[14] reported that IL–6 stimulated bone resorption and formation of osteoclasts in fetal mouse metacarpal and calvarial organ cultures, respectively. And Kurihara *et al.*[26] demonstrated the stimulatory effects of IL–6 on osteoclast formation in human marrow cultures. Taken together with the reports that IL–6 is also produced by osteoblasts, it is suggested that IL–6 may role as a possible mediator of bone resorptive action of systemic or local osteotropic agents. Therefore, we observed the regulation of IL–6 mRNA expression in human and rat osteoblastic cells by several systemic osteotropic hormones and cytokines, known to stimulate bone resorption and act indirectly via osteoblasts.

The steady state levels of IL–6 mRNA in MG–63 cells treated with various bone–resorbing agents are shown in Fig. 1. This result suggests that IL–6 is not produced in significant amounts in MG–63 cells except in response to exogenous stimuli. In these cells, neither PTH nor $1,25(OH)_2D_3$ induced IL–6 mRNA expression. These results are consistent with previous reports. Littlewood *et al.*[16] and Linkhart *et al.*[17] showed that PTH and $1,25(OH)_2D_3$ had no effect on IL–6 secretion in human osteoblast–like cells and Holt *et al.*[27] demonstrated that IL–6 did not mediate the stimulation by PTH, $1,25(OH)_2D_3$, or $PGE_2$ of osteoclast differentiation and bone resorption. In addition, previously we observed that PTH and $1,25(OH)_2D_3$ did not induce IL–6 secretion into culture medium in MG–63 culture[28]. Though Feyen *et al.*[11], Al–humidan *et al.*[12] and Holt *et al.*[29] reported that PTH, $1,25(OH)_2D_3$, and $PGE_2$ induced IL–6 release by mouse calvaria and mouse osteoblastic cells, these results, taken together with above reports, suggest that in bone resorbing action of PTH and $1,25(OH)_2D_3$, IL–6 may not be an essential component, at least in human.

On the contrary, treatment with IL–1$\beta$ or TNF–$\alpha$ greatly increased IL–6 mRNA expression in MG–63 cells, though induction potency of TNF–$\alpha$ was lesser than that of IL–1$\beta$ (Fig. 1). IL–6 expression was reported to be readily induced in many types of cells by the inflammatory mediators, IL–1 and TNF[30–36]. In addition, Al–Humidan *et al.*[12], Linkhart *et al.*[17], Littlewood *et al.*[16], and Shin *et al.*[28] also found that these local cytokines induced IL–6 production from mouse or human osteoblastic cells, taken together with our result, suggesting that increased production of IL–6 was due to increased amount of IL–6 mRNA. Though we could not explain from this data whether increase of IL–6 mRNA amounts was due to enhanced transcription or transcripts stabilization, it seems that these factors increase both transcription and mRNA stability according to previous reports[33,36].

When the time course of IL–6 induction was observed, IL–6 mRNA was detected at 6 hrs after adding IL–1$\beta$ or TNF–$\alpha$ and increased progressively up to 48 hrs (Fig. 2 and 3). These results were somewhat different from previous reports. Nemunaitis *et al.*[33], Elias and Lentz[36], and Linkhart *et al.*[17] reported that while maximum levels of IL−6 mRNA were observed at 8 − 12 hrs after stimulation by IL−1, TNF induce maximal expression of IL−6 at 30 min to 4 hrs after stimulation. Though the reason for the discrepancy is unknown, the recent report that TNF−$\alpha$ stimulated IL−6 expression in a biphasic manner, rapid early induction is followed by a decrease and then a second increase in mRNA levels[37], may suggest that in case of TNF, our data correspond to second phase induction of them, considering that we didn't determined IL−6 levels at immediate early periods after stimulation.

The dose−response effects of IL−1$\beta$ and TNF−$\alpha$ in inducing IL−6 mRNA expression in MG−63 cells are shown in FIg. 4 and 5. The expression of IL−6 mRNA were was dose−dependently increased by IL−1$\beta$ (Fig. 4). In TNF−$\alpha$ treated cells, the maximum expression of IL−6 mRNA was induced by 10 ng/ml of TNF−$\alpha$ and no more induction was observed in cells treated with higher concentration of TNF−$\alpha$. As described above, IL−6 induction potency of TNF−$\alpha$ was less than that of IL−1$\beta$ , and these results are consistent with the report of Littlewood *et al.*[16] and Shin *et al.*[28] They observed significant increase of IL−6 secretion by IL−1$\beta$ and TNF−$\alpha$ in human osteoblastic cells and TNF was also less potent than IL−1 in their culture. TNF was known to be generally 100−1,000 times less potent than IL−1 in bone *in vitro*[38].

IL−1 is widely accepted as a powerful bone resorbing cytokine[39−43] and this stimulatory effect is most likely due to promotion of proliferation and differentiation of hematopoietic progenitor cells of osteoclasts[44,45], similar to IL−6. Similar to IL−1 and IL−6, TNF is well known as a bone resorbing cytokine[46−49] and this effect of TNF is also probably due to increased osteoclast formation[24,44]. Recently Black *et al.*[50,51] reported that antibodies to IL−6 inhibited the effects of IL−1 or TNF on bone, together with aforementioned data, suggesting the role of IL−6 as a mediator of TNF action.

In addition to MG−63 cells, we observed the regulation of IL−6 mRNA expression in ROS 17/2.8 cells, rat osteoblastic cells. As the cDNA sequence coding for the mature portion of rat IL−6 is 68% identical with the corresponding human sequence[52], we used human IL−6 cDNA as a detecting probe. As in MG−63 cells, the basal expression of IL−6 mRNA was barely detectable in ROS 17/2.8 cells. Dissimilar to the results in MG−63 cells, however, the marked stimulation of IL−6 mRNA expression did not occurred in ROS 17/2.8 cells by any treatment with above bone resorbing agents when observed at 24 hrs after addition (data not shown). This result was somewhat different from previous reports in that PTH stimulates IL−6 secretion from primary rat calvarial cells or rat osteosarcoma−derived osteoblastic cells[13,16]. Although the reason for this discrepancy is unclear, recent report that PTH rapidly and transiently increased IL−6 mRNA levels, with maximal expression at 1 hr, in MC3T3−E1 osteoblastic cells[37] suggest that IL−6 mRNA level have declined to basal level at 24 hrs after PTH addition in our experiment.

To summarize, the present study suggests that elevation of IL−6 production in osteoblasts may be involved in bone resorbing activity of IL−1$\beta$ and TNF−$\alpha$, at least in human, and this enhanced production of IL−6 is due to increase in IL−6 transcripts level.

## V. CONCLUSION

To examine the possible role of IL−6 as a mediator of bone resorption stimulatory agents, MG−63 and ROS 17/2.8 cells, human and rat osteosarcoma derived osteoblastic cells, were cultured with several systemic and local bone−resorbing agents and their expression of IL−6 mRNA was determened. In both cells basal expression of IL−6 mRNA was barely detectable. When MG−63 cells were exposed to IL−1$\beta$ (1 ng/ml) or TNF−$\alpha$ (20 ng/ml) for 24 hrs, IL−6 mRNA levels greatly increased while PTH ($10^{-8}$ M) or $1,25(OH)_2D_3$ ($10^{-7}$ M) did not affect IL−6 mRNA levels. In MG−63 cells, IL−6 mRNA was detectable at 6 hrs poststimulation with either IL−1$\beta$ (1 ng/ml) or TNF−$\alpha$ (20 ng/ml), and its level increased progressively up to 48 hrs after stimulation. In addition, 0.1 to 10 ng/ml IL−1$\beta$ stimulated IL−6 expression in a dose−dependent manner and this stimulatory effect was more potent than that of TNF−$\alpha$. However, dissimilar to MG−63 cells, IL−6 mRNA levels in ROS 17/2.8 cells were not affected by exposure to above agents at 24 hrs. These results taken together suggest that elevation of IL−6 production in osteoblasts may be involved in bone resorbing activity of IL−1$\beta$ and TNF−$\alpha$, at least in human, and this enhanced production of IL−6 is due to increase in IL−6 transcripts level.

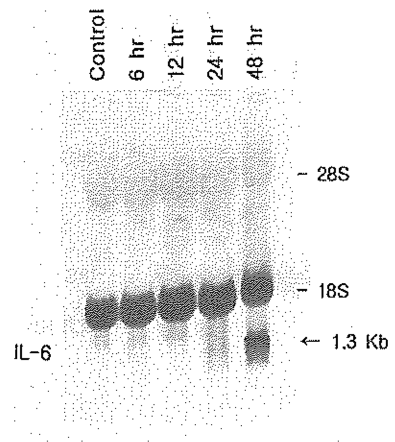


Fig. 2. Time course of change in the expression of IL−6 mRNA expression in MG−63 cells treated with IL−1$\beta$ After being cultured in serum−free medium for 24 hrs, cells were exposed for the indicated times to 1 ng/ml of IL−1$\beta$ Total cellular RNAs were extracted and subjected to northern blot analysis as described in Meterials and Methods.

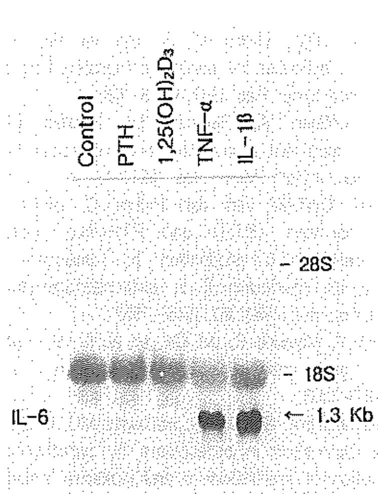


Fig. 1. Northern blot analysis of the IL−6 mRNA expression by various systemic and local bone resorbing agents in MG−63 cells. MG−63 cells were treated for 24 hrs without (control) or with $10^{-8}$ M of PTH, $10^{-7}$ M of $1,25(OH)_2D_3$, 20 ng/ml of TNF−$\alpha$ and 1 ng/ml of IL−1$\beta$ Total cellular RNAs were extracted and subjected to northern blot analysis as described in Materials and Methods.

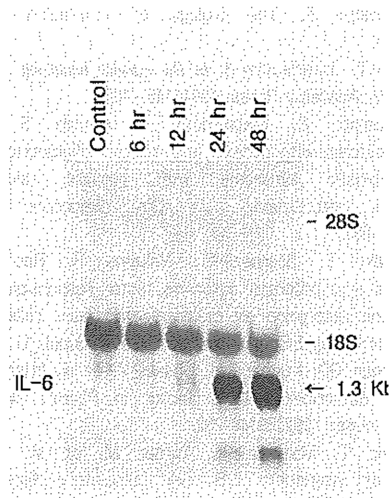


Fig. 3. Time course of change in the expression of IL−6 mRNA expression in MG−63 cells treated with TNF−$\alpha$ After being cultured in serum−free medium for 24 hrs, cells were exposed for the indicated times to 20 ng/ml of TNF−$\alpha$ Total cellular RNAs were extracted and subjected to northern blot analysis as described in Materials and Methods.

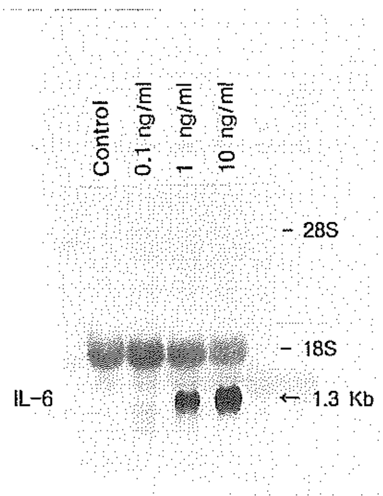


Fig. 4. Dose response effects of IL-1$\beta$ in inducing IL-6 mRNA expression of MG-63 cells. After being cultured in serum-free medium for 24 hrs, cells were treated for 24 hrs with graded concentrations of IL-1$\beta$ (ng/ml). Total cellular RNAs were extracted and subjected to northern blot analysis as described in Materials and Methods.

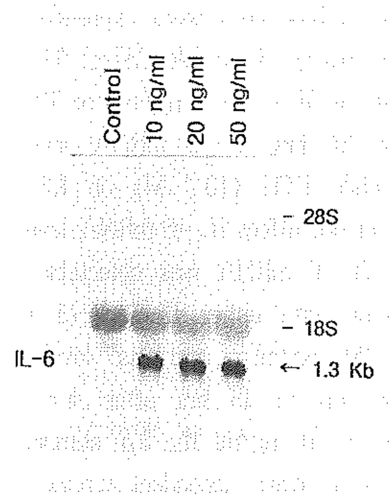


Fig. 5. Dose response effects of TNF-$\alpha$ in inducing IL-6 mRNA expression of MG-63 cells. After being cultured in serum-free medium for 24 hrs, cells were treated for 24 hrs with graded concentrations of TNF-$\alpha$ (ng/ml). Total cellular RNAs were extracted and subjected to northern blot analysis as described in Meterials and Methods.

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