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

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ABSTRACT

The aim of this study was to investigate the effects of bone resorbing agents on the expression of interleukin-6 (IL–6) in osteoblastic cells. Osteoblasts were treated with various concentrations of bone resorbing agents, and the expression of IL–6 was determined by Real-Time PCR. The results showed that IL–6 expression was significantly increased in a dose-dependent manner. These findings suggest that bone resorbing agents may play a role in the regulation of IL–6 expression in osteoblastic cells.

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, E cell growth and differentiation, immune responses, and acute-phase reactions\(^1\)\(^\text{–}^2\).

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 Ohboshi et al.\(^4\) demonstrated that excessive bone resorption seen in patients who have rheumatoid arthritis, giant cell tumors of bone is attributable to IL–6 secreted by tumor cells. Rodman et al.\(^5\) found that conditioned media from pathologic 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\(^6\)\(^\text{–}^7\). And the role of IL–6 has been demonstrated in osteoporosis.
associated with estrogen deficiency\textsuperscript{a,10} and alveolar bone destruction in periodontal disease as well\textsuperscript{9,10}. 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 \textit{et al.}\textsuperscript{11} and Al–Humidan \textit{et al.}\textsuperscript{12} reported that IL–6 was released from mouse bones in organ culture. Lowik \textit{et al.}\textsuperscript{13} and Ishimi \textit{et al.}\textsuperscript{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 \textit{et al.}\textsuperscript{11} and Greenfield \textit{et al.}\textsuperscript{15} demonstrated that parathyroid hormone (PTH) stimulated mRNA expression and secretion of IL–6 in murine osteoblastic cells. And Al–Humidan \textit{et al.}\textsuperscript{12} reported that interleukin–1\(\alpha\) (IL–1\(\alpha\)), PTH, and 1,25–dihydroxyvitamin D\(_3\) (1,25(OH)\(_2\)D\(_3\)) induced IL–6 release by mouse calvaria. Littlewood \textit{et al.}\textsuperscript{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 \textit{et al.}\textsuperscript{16} and Linkhart \textit{et al.}\textsuperscript{17} showed that PTH and 1,25 (OH)\(_2\)D\(_3\) 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)\(_2\)D\(_3\) was obtained from Calbiochem–Novochem 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 Mogun Biotechnology Research Institute (Yongin–Kun, Kyonggi–Do, Korea). \(\textsuperscript{32}\)P–dCTP, megaprime\textsuperscript{TM} DNA labelling system, and Hybond–N nylon membrane were purchased from Amersham Life Science (Arlington Heights, IL, USA). RNase\textsuperscript{TM} 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, Rockville, 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\(^{-4}\) M), 1,25(OH)\(_2\)D\(_3\) (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°C with a complementary DNA to human IL-6 labeled with 32P—dCTP by random primer extension (Megaprime DNA labelling kit) and purified using Bio—Spin chromatography column. Hybridizing solution contains 50% formamide / 10% dextran sulfate / 5X SSPE (0.15 M NaCl, 0.01 M Na2HPO4, 0.01 M EDTA) / 2.5X Denhardt’s solution / 100 μg/ml denatured salmon sperm DNA. The blots were washed twice with 5X SSPE for 15 min at 42°C, and analyzed by autoradiography. RNA loading equivalence 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)2D3 induced IL-6 mRNA expression in these cells. On the other hand, treatment with 20 ng/ml of TNF—a and 1 ng/ml of IL-1β greatly increased the expression of IL-6 mRNA. The potency of TNF—a was lower than that of IL-1β.

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-1β (Fig. 2) or TNF—a (Fig. 3). After being cultured in serum—free medium, the cells were treated for the indicated time periods with 1 ng/ml of IL-1β or 20 ng/ml of TNF—a. The expression of IL-6 mRNA increased slightly at 6 hrs after adding IL-1β or TNF—a and increased progressively up to 48 hrs.

The dose—response effects of IL-1β and TNF—a 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-1β or TNF—a. The expression of IL-6 mRNA was dose—dependently increased by IL-1β (Fig. 4). In TNF—a treated cells, the maximum expression of IL-6 mRNA was induced by 10 ng/ml of TNF—a and no more induction was observed in cells treated with higher concentration of TNF—a.

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 resorption15,16. 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. 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. 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)₂D₃ and local regulators such as IL-1 or TNF on bone resorption are at least partly mediated by osteoblast-derived soluble factors. 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. The majority of in vitro evidence suggests that IL-6 is an osteotropic factor whose predominant effects are on cells of the osteoclast lineage. Løwik et al. reported that IL-6 stimulated bone resorption and formation of osteoclasts in fetal mouse metacarpal and calvarial organ cultures, respectively. And Kurihara et al. 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 play a 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)₂D₃ induced IL-6 mRNA expression. These results are consistent with previous reports. Littlewood et al. and Linkhart et al. showed that PTH and 1,25(OH)₂D₃ had no effect on IL-6 secretion in human osteoblast-like cells and Holt et al. demonstrated that IL-6 did not mediate the stimulation by PTH, 1,25(OH)₂D₃, or PGE₂ of osteoclast differentiation and bone resorption. In addition, previously we observed that PTH and 1,25(OH)₂D₃ did not induce IL-6 secretion into culture medium in MG-63 culture. Though Feyen et al. Al-humaidan et al. and Holt et al. reported that PTH, 1,25(OH)₂D₃, and PGE₂ 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)₂D₃, IL-6 may not be an essential component, at least in human.

On the contrary, treatment with IL-1β or TNF-α greatly increased IL-6 mRNA expression in MG-63 cells, though induction potency of TNF-α was lesser than that of IL-1β (Fig. 1). IL-6 expression was reported to be readily induced in many types of cells by the inflammatory mediators, IL-1 and TNF. In addition, Al-humaidan et al., Linkhart et al., Littlewood et al., and Shin et al. 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.

When the time course of IL-6 induction was observed, IL-6 mRNA was detected at 6 hrs after adding IL-1β or TNF-α and increased progressively up to 48 hrs (Fig. 2 and 3). These
results were somewhat different from previous reports. Nemunaitis et al., Elas and Lentz, and Linkhart et al. 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-α stimulated IL-6 expression in a biphasic manner, rapid early induction is followed by a decrease and then a second increase in mRNA levels, 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-1β and TNF-α 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-1β (Fig. 4). In TNF-α treated cells, the maximum expression of IL-6 mRNA was induced by 10 ng/ml of TNF-α and no more induction was observed in cells treated with higher concentration of TNF-α. As described above, IL-6 induction potency of TNF-α was less than that of IL-1β, and these results are consistent with the report of Littlewood et al. and Shin et al. They observed significant increase of IL-6 secretion by IL-1β and TNF-α 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.

IL-1 is widely accepted as a powerful bone resorbing cytokine and this stimulatory effect is most likely due to promotion of proliferation and differentiation of hematopoietic progenitor cells of osteoclasts, similar to IL-6. Similar to IL-1 and IL-6, TNF is well known as a bone resorbing cytokine and this effect of TNF is also probably due to increased osteoclast formation. Recently Black et al. 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, 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. 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 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-1β and TNF-α, 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
determined. In both cells basal expression of IL-6 mRNA was barely detectable. When MG-63 cells were exposed to IL-1β (1 ng/ml) or TNF-α (20 ng/ml) for 24 hrs, IL-6 mRNA levels greatly increased while PTH (10^{-8} M) or 1,25(OH)_{2}D_{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-1β (1 ng/ml) or TNF-α (20 ng/ml), and its level increased progressively up to 48 hrs after stimulation. In addition, 0.1 to 10 ng/ml IL-1β stimulated IL-6 expression in a dose-dependent manner and this stimulatory effect was more potent than that of TNF-α. 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-1β and TNF-α, 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-1β. After being cultured in serum-free medium for 24 hrs, cells were exposed for the indicated times to 1 ng/ml of IL-1β. Total cellular RNAs were extracted and subjected to northern blot analysis as described in Materials 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)_{2}D_{3}, 20 ng/ml of TNF-α and 1 ng/ml of IL-1β. 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-α. After being cultured in serum-free medium for 24 hrs, cells were exposed for the indicated times to 20 ng/ml of TNF-α. Total cellular RNAs were extracted and subjected to northern blot analysis as described in Materials and Methods.
Fig. 4. Dose response effects of IL-1β 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-1β (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-α 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-α (ng/ml). Total cellular RNAs were extracted and subjected to northern blot analysis as described in Materials and Methods.

참고문헌

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