Stimulation by Parathyroid Hormone of Interleukin-6 and Leukemia Inhibitory Factor Expression in Osteoblasts: An Immediate-early Gene Response Induced by cAMP Signal Transduction*

Edward M. Greenfield†§¶, Mark C. Horowitz**, and Sandra A. Lavish‡

From the Department of Orthopaedics, the §Department of Physiology and Biophysics, and the ¶Institute of Pathology, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106-5000 and the **Department of Orthopaedics and Rehabilitation, Yale University School of Medicine, New Haven, Connecticut 06520-8071

*This work was supported in part by Research Grant 91-010 from the Orthopaedic Research and Education Foundation (to E. G.), a Pilot Study Grant from the Cuyahoga County Unit of the American Cancer Society (to E. G.), an Arthritis Foundation Fellowship (to E. G.), and National Institutes of Health Grants AR41674 (to E. G.), AR40073 (to M. H.), and AR40507 (to M. H.). Presented in part at annual meetings of the American Society for Bone and Mineral Research, Minneapolis, MN, September 30 to October 4, 1992, and Kansas City, MO, September 9–13, 1994, and of the American Society for Cell Biology, Denver, CO, November 15–19, 1992. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom correspondence and reprint requests should be addressed: Dept. of Orthopaedics, Case Western Reserve University, 11100 Euclid Ave., Cleveland, OH 44106-5000. Tel.: 216-368-1331; Fax: 216-368-1332.

Parathyroid hormone and other agents that stimulate bone resorption function, at least in part, by inducing osteoblasts to secrete cytokines that stimulate osteoclast differentiation and activity. We previously demonstrated that parathyroid hormone induces expression by osteoblasts of interleukin-6 and leukemia inhibitory factor without affecting the 16 other cytokines that were examined. We also showed that stimulation of osteoclast activity by parathyroid hormone is dependent on activation of the cAMP signal transduction pathway and secretion of interleukin-6 by osteoblasts. In the current study, we demonstrate that the rapid and transient stimulation of interleukin-6 and leukemia inhibitory factor is inhibited by actinomycin D and superinduced by protein synthesis inhibitors, the classical characteristics of an immediate-early gene response. Moreover, activation of cAMP signal transduction by parathyroid hormone and parathyroid hormone-related protein is necessary and sufficient to induce both interleukin-6 and leukemia inhibitory factor. In addition, cAMP analogues as well as vasoactive intestinal peptide and isoproterenol, two neuropeptides that stimulate bone resorption by activating cAMP signal transduction in osteoblasts, also induce interleukin-6 and leukemia inhibitory factor in these cells. Taken together with our previous results, this study suggests that interleukin-6 is crucial for stimulation of bone resorption not only by parathyroid hormone, but also by parathyroid hormone-related protein, vasoactive intestinal peptide, and β-adrenergic agonists, like isoproterenol.

Excessive skeletal loss is caused by a disturbance in the normal balance between bone resorption by osteoclasts, and bone formation by osteoblasts. Knowledge of the mechanisms that regulate these processes is fundamental to the understanding of the pathogenesis of bone loss that occurs in conditions such as osteoporosis. In addition to forming bone, osteoblasts appear to mediate the effect of parathyroid hormone (PTH) and other resorptive agents (1, 2). This concept is best supported by evidence that many agents, including PTH, stimulate bone resorption by osteoclasts that are cultured in the presence of osteoblasts but not by osteoclasts that are cultured alone. In most cases, resorption is also stimulated if the osteoblasts are replaced by conditioned media from osteoclasts activated by the resorptive agents. It is therefore thought that the primary effect of the resorptive agents is to stimulate osteoclasts to produce soluble cytokines that activate the osteoclasts.

We previously examined the effect of PTH on expression by osteoblasts of mRNAs encoding 18 cytokines, and found that only interleukin-6 (IL-6) and leukemia inhibitory factor (LIF) were stimulated by the hormone (3, 4). Maximal stimulation was approximately 50-fold for IL-6 and approximately 10-fold for LIF. PTH also stimulates secretion by osteoclasts of both IL-6 (3–9) and LIF (10) proteins. Lack of stimulation of IL-6 and LIF by PTH in particular cell preparations is therefore likely due to investigation of osteoclasts at different developmental stages or lack of sensitivity of the methods used. Thus, such results should not be interpreted to mean that PTH does not in general stimulate IL-6 or LIF in osteoblasts (reviewed in Ref. 3).

Our previous detailed time course studies showed that stimulation by PTH of IL-6 and LIF mRNA expression was both rapid and transient in MC3T3-E1 osteoblastic cells, primary rat osteoclasts, and in vivo osteoblasts (3, 11). IL-6 and LIF mRNA levels were maximal 30–60 min after stimulation and returned to baseline within 4–6 h; a pattern that is typical for immediate-early gene responses (12, 13). In the current study, we show that the stimulation of IL-6 and LIF by PTH is inhibited by actinomycin D and superinduced by protein synthesis inhibitors, the distinguishing characteristics of an immediate-early gene response (12, 13).

In conjunction with evidence that exogenous IL-6 and LIF can stimulate bone resorption (6, 8, 14–16), the realization that PTH stimulates IL-6 and LIF expression led to the suggestion that production of these cytokines might be involved in stimulation of resorption by a variety of agents, including PTH (3). In confirmation of this hypothesis, we have recently shown that an antibody directed against the IL-6 receptor can block osteoclast activation induced by PTH (17). The importance of IL-6...
is underscored by recent findings that implicate the cytokine in the increased bone resorption that occurs during estrogen withdrawal, Paget’s disease, rheumatoid arthritis, multiple myeloma, hypercalcemia of malignancy, hyperparathyroidism, and Gorham-Stout disease (reviewed in Ref. 18).

PTH-related protein (PTHrP), which is responsible for most cases of hypercalcemia of malignancy (19), also induces IL-6 secretion by osteoblasts (6, 7). PTHrP and PTH utilize the same receptor to activate both the classical adenyl cyclase pathway as well as the phospholipase C pathway (20, 21). Adenyl cyclase produces cAMP to activate protein kinase A, while phospholipase C produces inositol 1,4,5-trisphosphate and diacylglycerol to activate calcium-calmodulin-dependent kinase and protein kinase C, respectively. In order to fully understand the mechanism of action of PTH and PTHrP, it is important to clarify which signal transduction pathways are involved in the response of interest. PTH-(3-34), a truncated form of PTH, activates phospholipase C without stimulating cAMP production and can, therefore, be employed to determine whether cAMP signal transduction is involved in a particular response (22, 23). We (17) and others (22) have utilized this partial agonist to show that activation of the cAMP pathway in osteoblasts is required for stimulation of resorption by PTH. Other workers have reached the same conclusion using inhibitors of the cAMP pathway (24, 25).

Because stimulation of resorption by PTH depends on activation of the cAMP pathway and on IL-6 production, it is important to determine whether IL-6 production depends on cAMP signaling. Activation of either the protein kinase A, protein kinase C, or intracellular calcium pathways can stimulate IL-6 and LIF expression by osteoblasts (26) or other mesenchymal cells (27-30). Nonetheless, it is unknown which pathways are required for stimulation of IL-6 by PTH and PTHrP. In this study, we employed PTH-(3-34) to distinguish between these pathways. We show here that activation of the cAMP signaling pathway in osteoblasts is required for stimulation of IL-6 and LIF gene expression by PTH and PTHrP. Moreover, osteoblastic cAMP signaling is also required for stimulation of IL-6 and LIF by two other bone resorptive agents, namely vasoactive intestinal peptide (VIP) and isoproterenol.

MATERIALS AND METHODS

All reported data are representative of multiple experiments. PTH, PTHrP, VIP, and their analogues were obtained from Bachem (Torrance, CA); RNA and protein synthesis inhibitors, cAMP analogues, and β-adrenergic reagents were from Sigma. Of all of these agents were screened for endotoxin contamination using the colorimetric Limulus amoebocyte lysate assay (QCL-1000, Whittaker Bioproducts, Walkersville, MD). Endotoxin levels were $<0.0008$ EU/ml for the highest concentration of each hormone used in the experiments, $<0.015$ EU/ml for the highest concentration of 8-BrcAMP used, $<0.0075$ EU/ml for cycloheximide, and $<0.034$ EU/ml for puromycin. Moreover, none of the stimulatory agents contained endotoxin contamination detectable by our previously described (3, 17) functional assay of cytokine induction in MC3T3-E1 cells (see Fig. 9, for an example of this assay).

UMR106-01 rat osteblast-like osteosarcoma cells (31) were kindly provided by Dr. N. Partridge (St. Louis University). UMR106-01 cells between passage 18 and 24 were harvested using 0.02% EDTA (Sigma) and cultured in phenol-red free (32) minimum essential media (Life Technologies, Inc., Gaithersburg, MD) containing non-essential amino acids (Mediatech, Herndon, VA), 2 mM l-glutamine (Mediatech, Herndon, VA), 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT), 100 units/ml penicillin (Mediatech), and 100 μg/ml streptomycin (Mediatech). All media, serum, and additives were from lots that contained the lowest concentration of endotoxin available. For studies of immediate-early gene responses, confluent UMR106-01 cultures were made quiescent by incubation in serum-free media containing 1 mg/ml bovine serum albumin (AB412, Sigma) for 48 h prior to addition of inhibitors, stimulators, and vehicles. For signal transduction studies, stimulators and vehicles were added 2 h after plating (25,000 cells/cm²) UMR106-01 cells in order to mimic the conditions used in our previous studies of signal transduction during PTH-induced osteoclast activation (17). Murine MC3T3-E1 osteoblastic cells (32) were kindly provided by Dr. H. Tanaka (Okayama University) and experiments were performed 1 day after reaching confluence as described previously (3). In experiments using RNA or protein synthesis inhibitors, actinomycin D (10 μM), cycloheximide (10 μg/ml), or puromycin (100 μg/ml) were added immediately prior to addition of PTH. Spent media from both cell lines were consistently negative when assayed for mycoplasma contamination by solution hybridization to a probe complementary to mycoplasma rRNA (Gen-Probe, San Diego, CA).

IL-6 and LIF mRNA levels were assessed by RT-PCR as described previously (3). Murine IL-6 primers were previously described (3) and the rat IL-6 primers were modified versions of the mouse primers based on the published rat sequence (34). LIF primers were previously described (3) and were used for both mouse and rat, as they are based on the murine sequence (35) and have been used by other workers with UMR cells (36). Actin mRNA levels were employed in all experiments as a constitutive control using the PCR primers described in Ref. 37. All of the PCR primer pairs flanked at least one intron to avoid false positives due to amplification of genomic DNA. Controls without cDNA and with known positive cDNAs were used in all PCR reactions. To confirm the identity of the PCR products, we showed that fragments of appropriate sizes were produced by diagnostic restriction enzymes (HpaII, MboI, and HpaI for IL-6, LIF, and actin, respectively) as described previously (3).

IL-6 protein was determined in supernatants harvested 24 h after addition of stimulators or vehicles. In these studies, cultures were rinsed twice with phosphate-buffered saline before adding stimulators and vehicles dissolved in the media described above except containing 0.5% fetal bovine serum. IL-6 bioactivity was assessed by measuring proliferation of the B cell hybridoma B13.29 (also known as B9) as described previously (3, 7). Purified recombinant murine IL-6 (R&D Systems, Minneapolis, MN) was used in each bioassay to establish a standard curve and serve as a positive control. The presence of IL-6 was confirmed by preincubation of the culture supernatants for 30-40 min prior to addition of the B9 cells with MFS-17D2, a neutralizing rat monoclonal anti-murine IL-6 antibody obtained from Dr. J. Abrams (DNAX Research Institute) (38).

RESULTS

Stimulation of IL-6 and LIF Expression by PTH Is an Immediate-early Gene Response—We previously showed that stimulation by PTH of IL-6 and LIF mRNA expression is rapid and transient in the MC3T3-E1 osteoblast cell line, primary rat osteoblasts, and osteoblasts in vivo (Refs. 3 and 11, also see Figs. 2 and 3). Levels of both IL-6 and LIF mRNAs peak 30-60 min after addition of PTH and return to baseline by 4-6 h. Since such kinetics are reminiscent of immediate-early responses (12, 13), we examined whether induction of IL-6 and LIF by PTH exhibits other characteristics of classical immediate-early responses. Immediate-early responses depend on increased transcription rather than mRNA stability and are, therefore, inhibited by transcription blockers, such as actino-

2 S. A. Lavish and E. M. Greenfield, unpublished observations.
mycin D (12, 13). Fig. 1 shows that stimulation of IL-6 and LIF mRNA expression by PTH is inhibited by actinomycin D and that PTH has no detectable effect on stability of either cytokine mRNA. Although these results are consistent with an immediate-early response, conclusive evidence requires examination of protein synthesis inhibitors. Because immediate-early responses are primary effects that do not require synthesis of protein intermediate proteins, they are not blocked by inhibition of protein synthesis (12, 13). Instead, immediate-early responses are superinduced; that is, they are increased in both duration and magnitude (12, 13). Although cycloheximide is the classical protein synthesis inhibitor used in studies of immediate-early responses, it has recently been shown to activate the stress-activated protein kinase signal transduction pathway (39, 40). Puromycin is a protein synthesis inhibitor with a distinct mode of action (41) that does not activate stress-activated protein kinase signal transduction (40). We therefore investigated the effects of both cycloheximide and puromycin. The two protein synthesis inhibitors gave indistinguishable results in both MC3T3-E1 and UMR106-01 cells. For example, Fig. 2 shows the effect of cycloheximide on MC3T3-E1 cells and Fig. 3 shows the effect of puromycin on UMR106-01 cells. In all cases, inhibition of protein synthesis superinduces the PTH-dependent stimulation of IL-6 and LIF mRNAs. In comparison, the inhibitors have only modest effects in the absence of PTH (Figs. 2 and 3). Taken together with the rapid and transient kinetics and the inhibition caused by actinomycin D, these results demonstrate that stimulation of IL-6 and LIF by PTH is a classical immediate-early gene response.

cAMP Signal Transduction Is Necessary and Sufficient for Stimulation of IL-6 and LIF Expression by PTH—Fig. 4 demonstrates that IL-6 and LIF mRNAs are stimulated by PTH in a dose-dependent manner; stimulation is detectable with concentrations as low as 0.025 nM PTH and is maximal at 25 nM. PTHrP also stimulates IL-6 and LIF mRNAs (Fig. 4) with a similar dose dependence (data not shown). To assess if cAMP-mediated signal transduction is required for stimulation of IL-6 and LIF mRNA levels by PTH and PTHrP, we examined whether the effects of these agents are mimicked by PTH-(3–34). This partial agonist has no detectable effect on expression of IL-6 and LIF mRNAs even at 100 nM (Fig. 4). Since PTH-(3–34) activates phospholipase C but not adenyl cyclase (23, 42), these results demonstrate that cAMP signal transduction is required for stimulation of IL-6 and LIF mRNA expression by PTH and PTHrP.

To further study the role of cAMP, we examined whether membrane-permeant analogues of cAMP effect IL-6 and LIF mRNA levels. Fig. 5 shows that 30–300 μM 8-Br-cAMP dose dependently stimulates expression of IL-6 and LIF. In contrast, 300 μM 8-Br-cGMP, which was used as a negative control, has no detectable effect (Fig. 5). Expression of IL-6 and LIF is also stimulated by both mono- and dibutyryl-cAMP but not by the inactive analogue, monobutyril cyclic deoxyadenosine monophosphate (data not shown).

To determine whether receptor-mediated intracellular cAMP is sufficient to induce IL-6 and LIF mRNA expression, we asked whether the cytokines are also induced by other bone resorptive agents that also stimulate cAMP in osteoblasts, namely VIP (43) and isoproterenol (44). VIP dose dependently stimulates expression of IL-6 and LIF (Fig. 6), with detectable and maximal effects at 0.1 and 10 nM VIP, respectively. The same concentrations of [4C]-o-Phe6,Leu21VIP (VIP-A), a weak VIP agonist (45), have lesser effects on the level of either cytokine mRNA (Fig. 6). Although VIP is thought to function primarily through the cAMP signal transduction system (46), it can also stimulate other signaling pathways in certain cell types (47–49), possibly including osteoblasts (50, 51). To avoid
this potential complication, we examined the effect of the β-adrenergic agonist, isoproterenol, which specifically stimulates cAMP signal transduction (52). Isoproterenol dose-dependently stimulates expression of both IL-6 and LIF mRNAs (Fig. 7), with detectable and maximal effects at 0.1 and 10 μM, respectively. In contrast, the same concentrations of propranolol, a β-adrenergic antagonist which served as a negative control, did not stimulate expression of either cytokine (Fig. 7). In order to assess whether the increases in IL-6 mRNA illustrated in Figs. 4–7 are reflected in augmented IL-6 protein secretion, we employed the B9 hybridoma bioassay in conjunction with an antibody that neutralizes IL-6. We previously demonstrated that PTH-(1–34) stimulates IL-6 secretion using this method (3). Fig. 8 shows that all of the agents that stimulate expression of IL-6 mRNA (PTH-(1–34), PTHrP, 8-Br-cAMP, VIP, and isoproterenol) also induce secretion of IL-6 protein. Moreover, comparison to a standard curve constructed using purified recombinant murine IL-6 allowed estimation of the amount of IL-6 in each sample. These values were 0.14 ± 0.009 pg/ml for the control cultures, 77.2 ± 3.4 pg/ml for the PTH-treated cultures, 38.9 ± 2.4 pg/ml for the PTHrP-treated cultures, 35.0 ± 1.0 pg/ml for the 8-Br-cAMP-treated cultures, 4.2 ± 0.04 pg/ml for the VIP-treated cultures, and 3.5 ± 0.1 pg/ml for the isoproterenol-treated cultures. As expected, no effect was observed when the resorptive agents were added directly to the IL-6 bioassay (data not shown). Importantly, the IL-6 bioactivity secreted in response to these agents was reduced by >93% by an antibody that specifically neutralizes authentic IL-6 (data not shown). In contrast, an anti-IL-11 antibody (1H3/19.6.1 provided by Genetics Institute) had no detectable effect on the bioactivity in the media from cultures that had been treated with the resorptive agents (data not shown).

The results depicted in Figs. 4–8 show results with UMR106-01 cells to provide compatibility with our previous work demonstrating that stimulation of osteoclast activity by PTH depends on cAMP and IL-6 (17). The same series of stimulators and controls were employed to determine whether the cAMP signal transduction pathway is also essential for induction of IL-6 and LIF mRNA in MC3T3-E1 cells (Fig. 9). As was seen in UMR106-01 cells, all of the agents that are potent stimulators of cAMP signaling (PTH-(1–34), PTHrP, VIP, 8-Br-cAMP, and isoproterenol) strongly increase expression of IL-6 and LIF mRNAs, while the control compounds (PTH-(3–34), VIP-A, 8-Br-cGMPP, and propranolol) had little or no effect on expression of IL-6 and LIF mRNAs. Fig. 9 also shows that none of these agents detectably stimulate tumor necrosis factor α mRNA. Thus, stimulation of IL-6 and LIF mRNAs by these agents cannot be attributed to endotoxin contamination, since endotoxin strongly stimulates expression of tumor necrosis factor α mRNA (Fig. 9). This finding confirms the results of the colorimetric endotoxin assay described under “Materials and Methods.”

DISCUSSION

We previously showed that PTH rapidly and transiently stimulates expression of IL-6 and LIF mRNAs (3, 11), reminiscent of classical immediate-early responses (12, 13). The cur-
CAMP Stimulates Immediate-early Expression of IL-6 and LIF

These results are consistent with the demonstration that 1-alanyl-2-methylglycerol, a specific protein kinase C inhibitor, does not block the ability of PTH to stimulate IL-6 secretion (71). We cannot rule out the possibility that the inhibitors were not fully effective and that residual calcium or protein kinase C signal transduction may have been involved in stimulation of IL-6 and LIF by PTH. Nonetheless, the current study demonstrates that if the phospholipase C pathways are involved, they act together with CAMP.

Takagi and his coworkers have previously demonstrated that IL-6 is involved in stimulation of osteoclast activity by PTH (17), this study suggests that IL-6 is also involved in stimulation of resorption by PTHrP, VIP, and β-adrenergic agonists. All of these agents are thought to stimulate resorption through activation of osteoblast CAMP signal transduction (19, 43, 44). VIP is particularly interesting in this regard, since high concentrations of this molecule are found near focal bone resorption sites induced by tooth movement (72). Thus, in response to mechanical forces, sympathetic nerves innervating bone and periosteum (73) may secrete VIP, which would stimulate production by osteoblasts of factors such as IL-6 that regulate bone resorption. It has also been proposed that VIP may be involved in the stimulation of bone resorption observed in reflex sympathetic dystrophy (74). Similarity of function between PTH and VIP likely reflect homology between the intracellular signaling domains of the two receptors (75). In fact, it has recently been reported that PTH and VIP antagonists are able to inhibit activation of each other’s receptor (76).

In summary, this study demonstrates that induction of IL-6 and LIF by PTH is an immediate-early gene response that depends on CAMP signal transduction. Moreover, PTHrP, VIP, and isoproterenol also induce IL-6 and LIF expression through CAMP signal transduction. These results suggest that stimulation of osteoclast activity by PTHrP, VIP, and isoproterenol, like that by PTH, is mediated by production of IL-6 and possibly LIF. We and others have also shown that many resorptive agents that activate signaling pathways that do not involve CAMP also stimulate production of IL-6 by osteoblasts. These include IL-1, IL-6, LIF, tumor necrosis factor, platelet-derived growth factor, 1,25-dihydroxyvitamin D₃, transforming growth factor β, endothelin, and lipopolysaccharide (reviewed in Ref. 18). IL-1, IL-6, tumor necrosis factor, transforming growth factor β, and lipopolysaccharide have also been shown to stimulate LIF expression by osteoblasts (3, 4, 10, 16, 17, 36, 77). Thus, production of IL-6, and perhaps LIF, may be critically involved in stimulation of resorption by a wide variety of agents in addition to those examined in this study that activate CAMP signal transduction. Alternatively, LIF may be involved in stimulation of bone formation by PTH (78) since LIF increases osteoblastic differentiation (36, 79) while IL-6 has been reported to inhibit this process (80).

Acknowledgments—We thank Cathleen Carlin and Charles Malemud for many helpful discussions and Dylan Distasio for performing the IL-6 bioassays.

REFERENCES

1. Rodan, G. A., and Martin, T. J. (1981) Calif. Tissue Int. 33, 349–351
2. Horowitz, M. C. (1993) Science 260, 626–627
3. Greenfield, E. M., Gornik, S. A., Horowitz, M. C., Donahue, H. J., and Shaw, S. M. (1993) J. Bone Miner. Res. 8, 1163–1171
4. Greenfield, E., Blaha, M., and Merrill, M. (1996) Trans. Orthop. Res. Soc. 21, 337
5. Feyen, J. H. M., Eiford, P., Padova, F. E. D., and Trechsel, U. (1989) J. Bone Miner. Res. 4, 633–638
6. Lowik, C., van der Pluijm, G., Bloos, H., Hoekman, K., Bijvoet, O., Aarden, L., and Papapoulos, S. (1989) Biochem. Biophys. Res. Commun. 162, 1546–1552
7. Horowitz, M., Brown, M., Insogna, K., Cederman, D., Centrella, M., Phillips, J., and Weir, E. (1990) in Molecular and Cellular Biology of Cytokines (Opperhein, J. J., ed) pp. 471–476, Wiley-Liss, New York
8. Ishimi, Y., Miyaura, C., Jin, C. H., Akatsu, T., Abe, E., Nakamura, Y., Yamag-
Stimulation by Parathyroid Hormone of Interleukin-6 and Leukemia Inhibitory Factor Expression in Osteoblasts Is an Immediate-early Gene Response Induced by cAMP Signal Transduction
Edward M. Greenfield, Mark C. Horowitz and Sandra A. Lavish

J. Biol. Chem. 1996, 271:10984-10989.
doi: 10.1074/jbc.271.18.10984

Access the most updated version of this article at http://www.jbc.org/content/271/18/10984

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 73 references, 22 of which can be accessed free at http://www.jbc.org/content/271/18/10984.full.html#ref-list-1