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Adrenergic stimulation of osteoclastogenesis mediated by expression of osteoclast differentiation factor in MC3T3-E1 osteoblast-like cells

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Abstract

It is well known that adrenergic agonists efficiently activate β-adrenoceptors on osteoblastic cells and can stimulate bone resorption in intact mouse calvaria. Recently, an osteoclastogenic factor of osteoblastic origin was found to be a novel tumor necrosis factor ligand family member and was termed osteoclast differentiation factor (ODF). Using a reverse transcription–polymerase chain reaction approach, we investigated the effect of epinephrine on mRNA levels of ODF and its decoy receptor, osteoclastogenesis inhibitory factor (OCIF), in MC3T3-E1 cells. Treatment with epinephrine (1 μM) rapidly increased ODF and OCIF mRNA levels, which peaked after 0.5 hr of treatment. Epinephrine (1 μM) also increased interleukin (IL)-6, IL-11, and cyclooxygenase (COX)-II mRNA levels, as well as increased prostaglandin E_2 (PGE_2) accumulation in the culture medium. Treatment of the cells with IL-11 (10 ng/mL) or PGE_2 (1 μM) increased ODF and OCIF mRNA levels as observed with epinephrine. However, increases in ODF and OCIF mRNA levels by epinephrine were more rapid than those by IL-11, and were not influenced by NS-398 (100 μM; an inhibitor of COX-II), suggesting a direct effect of epinephrine on ODF and OCIF mRNA expressions as well as an indirect effect mediated by IL-11 and PGE_2 production. Epinephrine-induced increases in ODF and OCIF mRNA levels were inhibited by pretreatment with timolol (1 μM; β-antagonist) and phentolamine (1 μM; α-antagonist), respectively. Furthermore, the formation of tartrate-resistant acid phosphatase-positive multinucleated cells from mouse bone marrow cells was stimulated by isoproterenol (0.1 to 10 μM) or epinephrine (0.1 to 10 μM). The action of isoproterenol, a β-agonist, was clearly stronger than that of epinephrine, suggesting the importance of the physiological balance between ODF and OCIF productions for osteoclastogenesis. These findings suggest that β-adrenergic stimulation induces not only IL-6, IL-11, and PGE_2 but also ODF expression in osteoblastic cells, leading to a stimulation of osteoclastogenesis.

Keywords: Adrenergic agonist; Osteoblastic cell; Osteoclast differentiation factor; Osteoclastogenesis inhibitory factor; RT–PCR; Bone marrow culture

1. Introduction

In the bone microenvironment, there is a dynamic balance between resorption and formation that maintains skeletal homeostasis. Osteoclastic bone resorption consists of multiple steps such as the differentiation of osteoclast precursors into mononuclear prefusion osteoclasts, the fusion of prefusion osteoclasts to form multinucleate osteoclasts, and the activation of these osteoclasts to resorb bone [1,2]. These steps seem to progress at the site of bone resorption under the control of osteotrophic hormones locally produced in the microenvironment [1,3]. Potential paracrine mediators of osteoclast activity include M-CSF (monocyte-macrophage colony-stimulating factor), TNF-α, IL-1, IL-6, IL-11, and PGE_2, all of which are capable of increasing osteoclastogenesis.

Recently, two molecules produced by osteoblast lineage cells were identified to play important roles in osteoclastogenesis. One of these molecules is ODF, which is identical to OPGL, TRANCE (TNF-related activation-induced cytokine), and RANKL (receptor activator of nuclear factor-κB ligand). ODF/OPGL is a membrane-associated protein, and a genetically engineered soluble form bearing the extracellular domain of the protein induced osteoclast-like cell formation from spleen cells in the absence of osteoblasts/stromal cells [4–7]. The other molecule is OPG [8], which

Abbreviations: ODF, osteoclast differentiation factor; RT–PCR, reverse transcription–polymerase chain reaction; OCIF, osteoclastogenesis inhibitory factor; IL, interleukin; COX, cyclooxygenase; PGE_2, prostaglandin E_2; TRACP, tartrate-resistant acid phosphatase; MNC, multinucleated cell; TNF, tumor necrosis factor; OPGL, osteoprotegerin ligand; hPTH, human parathyroid hormone; α-MEM, α-modified minimum essential medium; and G3PDH, glyceraldehyde 3-phosphate dehydrogenase.

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is identical to OCIF [9]. OPG/OCIF is a secretory protein belonging to the TNF receptor family [8,9]. This protein inhibits not only formation of osteoclast-like cells in culture, but also bone resorption both *in vitro* and *in vivo* [8,10]. In recent experiments, the gene expression of both ODF and OCIF in osteoblasts/stromal cells was reported to be regulated by potent osteoclast-like cell formation-stimulating factors including $1,25(OH)_2D_3$, IL-11, PGE_2, and PTH [1,3].

Adrenergic receptors are well known to be present in osteoblastic cells, and the pharmacological activation of α- and β-receptors has been reported to stimulate the proliferation of these cells [11] and to cause bone resorption in an organ culture system [12], respectively. To gain a better understanding of the bone resorption induced by epinephrine, we examined the involvement of ODF and/or OCIF in epinephrine-induced bone resorption by determining the effect of epinephrine on the mRNA expression of ODF and OCIF in the mouse osteoblastic cell line MC3T3-E1. The present study shows that activation of the β-receptors increased ODF expression, whereas activation of α-receptors increased OCIF expression, and that β-agonists caused osteoclastogenesis in mouse bone marrow cells.

2. Materials and methods

2.1. Materials

MC3T3-E1 cells, established from newborn mouse calvaria by Kodama *et al*. [13], were supplied by Dr. Amagai (Oou University, Koriyama, Japan). α-MEM was obtained from GIBCO BRL and FBS (fetal bovine serum) from Cell Culture Laboratories. Tissue culture dishes were obtained from Falcon Plastics. Epinephrine, isoproterenol, phentolamine, and timolol were from Sigma Chemical Co. NS-398, an inhibitor of COX-II, was supplied by Taisho Pharmaceutical Co. All other chemicals used were of reagent grade.

2.2. Cell culture of MC3T3-E1 cells

Cloned MC3T3-E1 cells were cultured in plastic dishes in α-MEM supplemented with 10% FBS, 100 IU/mL of penicillin, and 100 μg/mL of streptomycin at 37° in air with 5% CO_2 and were then subcultured until almost confluent. For the experiments examining gene expression, cells were plated at 10^6 cells per 10-cm diameter dish, and confluent cell cultures were used in all experiments. PGE_2 was determined with a commercially available ELISA kit (Cayman Chemical).

2.3. Analysis of mRNA levels by RT–PCR

RNA was extracted from osteoblast-like cells in a 10-cm dish by the guanidium isothiocyanate method [14]. RT–PCR was performed by standard methods, as reported previously [15]. Briefly, at first cDNA was synthesized by use of random primers and Moloney murine leukemia virus reverse transcriptase (GIBCO BRL), followed by PCR amplification using synthetic gene primers specific for mouse ODF, mouse OCIF, and mouse G3PDH produced from their respective reported cDNA sequences [4,16–20]. The oligonucleotide primers were synthesized on a DNA synthesizer (Expedite model 8909; PerSeptive Biosystems) and purified by polypropylene filter (Oligo Prep Kit; Pharmacia Biotech). The following primers were used: ODF forward 21-

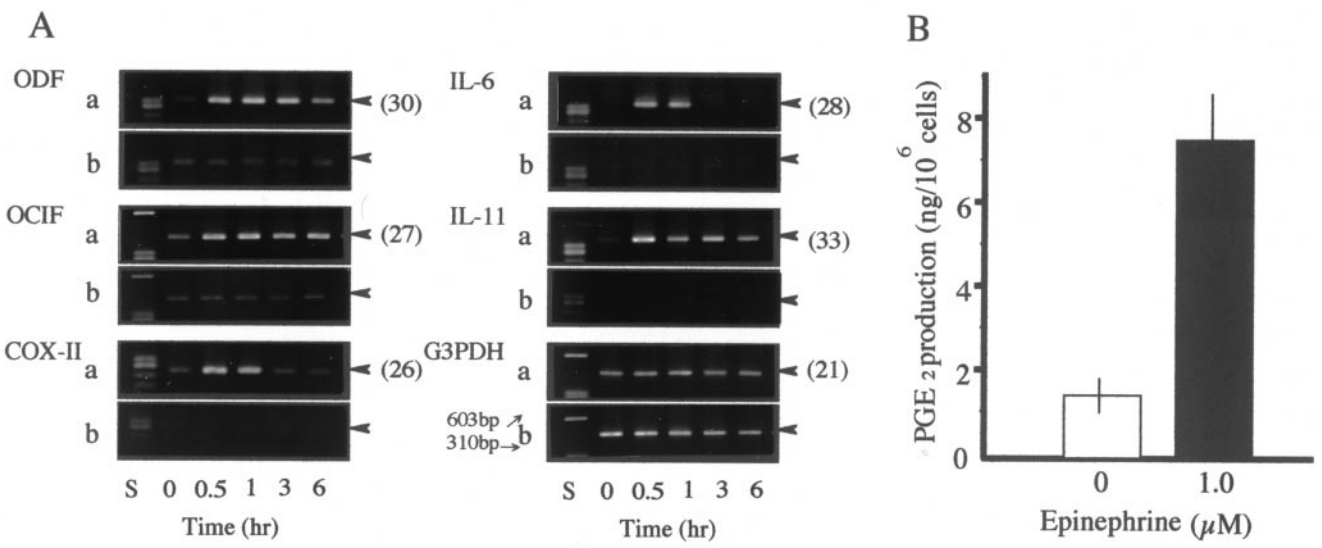

Fig. 1. Effect of epinephrine on induction of ODF, OCIF, COX-II, IL-6, and IL-11 mRNAs (A) and on accumulation of PGE_2 (B) in MC3T3-E1 cells. (A) RT–PCR analysis for mRNA obtained from cells treated with epinephrine (1 μM) (a) or vehicle (b) for 0.5, 1, 3, and 6 hr. Epinephrine was dissolved in α-MEM. DNA size markers ($\Phi X174/HaeIII$ digest) are shown in the left lanes (S). Numbers in parentheses on the right indicate cycles of PCR amplification. Arrowheads indicate the predicted sizes of PCR products. (B) PGE_2 accumulation in culture medium was determined by ELISA analysis at 12 hr after treatment with epinephrine (1 μM). Data are expressed as the means ± SEM of three cultures.

mer, 5′-GCCATTTGCACACCTCACCAT-3′; ODF reverse 21-mer, 5′-AGAATTGCCCGACCAGTTTTT-3′; OCIF forward 21-mer, 5′-CCTTGCCCTGACCACTCT TAT-3′; OCIF reverse 21-mer, 5′-ACATCTATTCCA CACTTTTGC-3′; IL-6 forward 21-mer, 5′-GAAAT GAGAAAAGAGTTGTGC-3′; IL-6 reverse 21-mer, 5′-ATTGGAAATTGGGGTAGGAAG-3′; IL-11 forward 21-mer, 5′-CACCATCGATACCGCCCTTTA-3′; IL-11 reverse 21-mer, 5′-CTTCCCTCCTTCCTCCTCTTA-3′; COX-II forward 21-mer, 5′-CTGGTGCCTGGTCTGATGATG-3′; COX-II reverse 21-mer, 5′-GGCAATGCGGTTCT GATACTG-3′; G3PDH forward 20-mer, 5′-ACCACAGTC CATGCCATCAC-3′; G3PDH reverse 20-mer, 5′-TCCAC CACCCTGTTGCTGTA-3′. PCR amplification was performed with a GeneAmp PCR System (Perkin Elmer/Cetus) according to the following schedule: denaturation at 95° for 30 sec, annealing at 55° for 30 sec, and elongation at 72° for 1 min for the appropriate number of cycles. PCR products were electrophoresed on a 2% Nusive GTG agarose gel (FMC BioProducts) and stained with ethidium bromide. All PCR reactions resulted in the amplification of a single product of the predicted size for ODF (316 bp), OCIF (407 bp), IL-6 (324 bp), IL-11 (344 bp), COX-II (255 bp), and G3PDH (452 bp), products that were detected with a FluorImager 575 (Molecular Dynamics). All PCR data were obtained from measurements performed in the linear range of PCR amplification.

2.4 Mouse bone marrow cultures

Mouse bone marrow cells were obtained from tibiae of 7- to 9-week-old male ddY mice as previously described [21]. Marrow cells were cultured in α-MEM containing 10% fetal bovine serum at 1.5×10^6 cells/mL in 24-well plates (1 mL/well). Cultures were fed every 3 days by

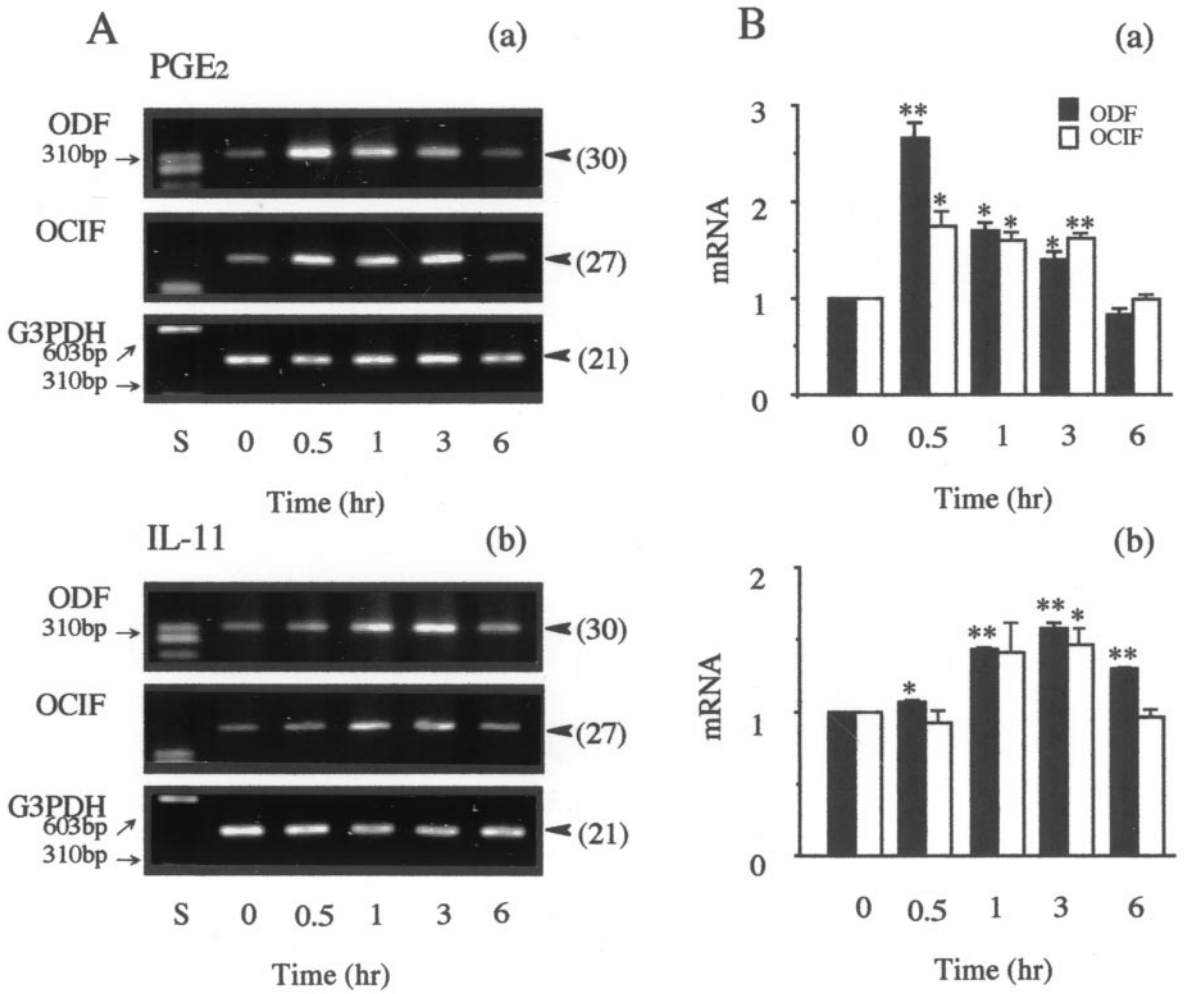

Fig. 2. Time-course of the effects of PGE_2 and IL-11 on induction of ODF and OCIF mRNAs in MC3T3-E1 cells. (A) RT–PCR analysis obtained from cells treated with PGE_2 (1 μM) (a) or IL-11 (10 ng/mL) (b) for 0.5, 1, 3, and 6 hr. DNA size markers (Φ*X174/HaeIII* digest) are shown in the left lanes (S). Numbers in parentheses on the right indicate cycles of PCR amplification. Arrowheads indicate the predicted sizes of PCR products. (B) Relative expression of ODF and OCIF mRNAs in response to PGE_2 (a) or IL-11 (b). The mRNA levels of ODF and OCIF were calculated by dividing the intensity of the ODF and OCIF bands by the intensity of the G3PDH band as determined by a fluorescence image analyzer. Pooled data from six independent experiments are expressed as means ± SEM ($^*P < 0.05$, $^{**}P < 0.01$ Student's *t*-test).

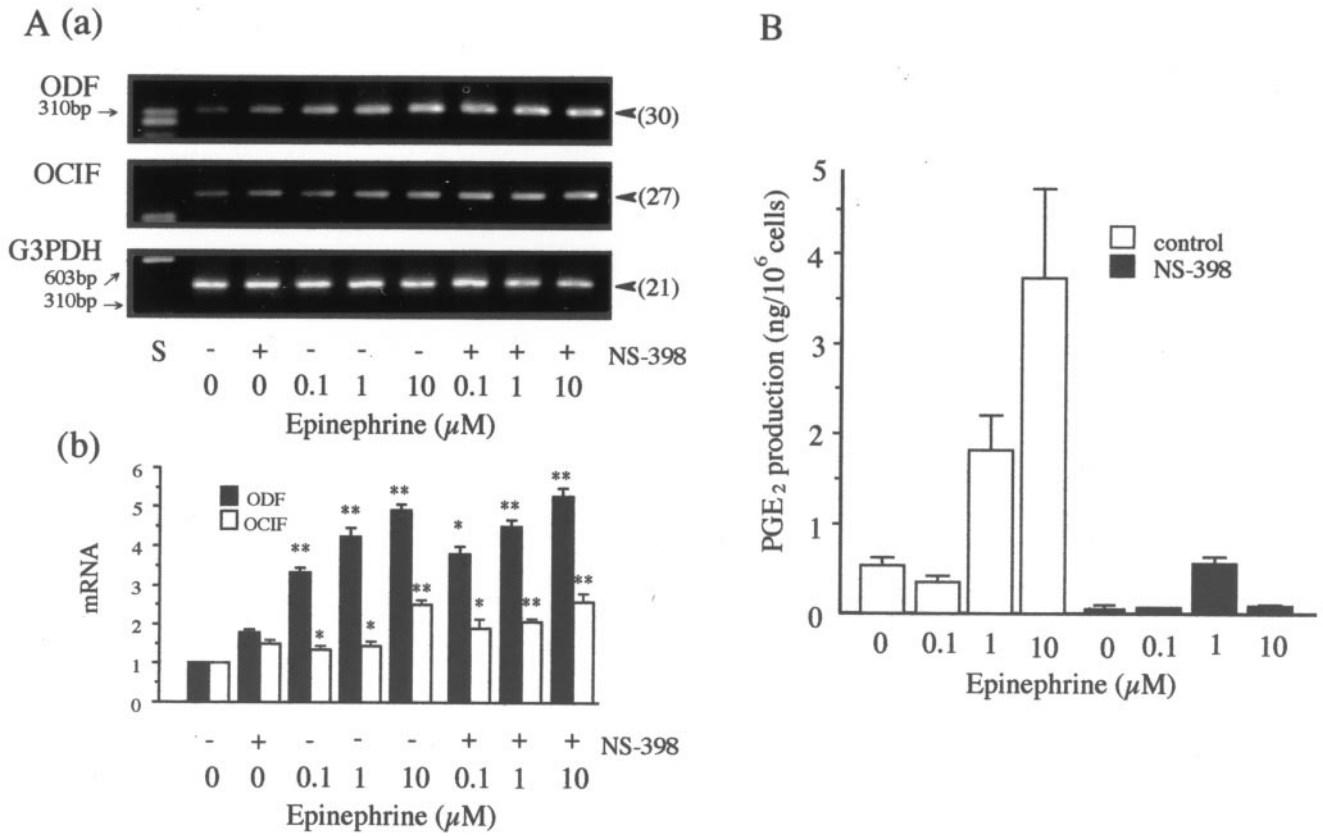

Fig. 3. Effect of NS-398 on induction of ODF and OCIF mRNAs (A) and on accumulation of PGE_2 (B) in MC3T3-E1 cells treated with epinephrine. (A) RT–PCR analysis (a) obtained from cells treated with epinephrine (0.1 to 10 μM) for 1 hr in the presence or absence of NS-398 (100 μM). NS-398 was treated 30 min before the treatment with epinephrine. DNA size markers (Φ*X174/HaeIII* digest) are shown in the left lanes (S). Numbers in parentheses on the right indicate cycles of PCR amplification. Arrowheads indicate the predicted sizes of PCR products. (b) Relative expression of ODF and OCIF mRNAs in response to epinephrine (1 μM) in the presence or absence of NS-398 (100 μM). The mRNA levels of ODF and OCIF were calculated by dividing the intensity of the ODF and OCIF bands by the intensity of the G3PDH band as determined by a fluorescence image analyzer. Pooled data from six independent experiments are expressed as means ± SEM (*$P < 0.05$, **$P < 0.01$ Student's *t*-test). (B) PGE_2 accumulation in culture medium was determined by ELISA analysis at 6 hr after treatment with epinephrine (0.1 to 10 μM) in the presence or absence of NS-398 (100 μM). NS-398 was treated 30 min before the treatment with epinephrine. Data are expressed as the means ± SEM of three cultures.

replacing 0.5 mL old medium with fresh medium. All cultures were maintained in a humidified atmosphere of 5% CO_2 in air at 37°. To examine the number of TRACP-positive MNCs, we added epinephrine or isoproterenol at the beginning of cultures and at each change of medium. After having been cultured for 7 days, adherent cells were rinsed with PBS, fixed with ethanol–acetone (50:50, v/v) for 1 min, and stained for TRACP by incubating the cells in 0.1 M sodium acetate buffer (pH 5.0) containing naphthol AS-MX phosphate and red violet LB salt in the presence of 10 mM sodium tartrate, as described previously [22]. Cells containing three or more nuclei were counted as MNCs. The results were expressed as the means ± SEM of quadruplicate cultures.

3. Results

This is the first report to demonstrate that osteoblasts are able to express ODF and OCIF mRNAs following stimulation by epinephrine. Fig. 1 shows the time-course of the effect of epinephrine on the induction of ODF, OCIF, COX-II, IL-6, and IL-11 mRNAs in MC3T3-E1 as examined by semiquantitative PCR. There was almost no detection of these mRNAs at the steady state under the experimental conditions used, but their mRNA levels were rapidly increased by treatment of the cells with epinephrine (1 μM). The expression of ODF and OCIF mRNAs was found to be maximal at 0.5 hr, and declined after 6 hr of treatment. As shown in Fig. 1B, the increased gene expression of COX-II caused a significant increase in its enzyme product, PGE_2, in the culture medium. Thus, epinephrine induced not only IL-6, IL-11, and PGE_2, which are known to stimulate osteoclastogenesis, but also ODF in MC3T3-E1 cells.

Both ODF and OCIF mRNA levels were increased by treatment with PGE_2 or IL-11, as observed by epinephrine, but not by IL-6. Fig. 2 shows the time-course of the effect of PGE_2 and IL-11 on induction of ODF and OCIF mRNAs in MC3T3-E1 cells. The time-course of the effect of PGE_2 was similar to that of epinephrine. PGE_2 (1 μM) caused a

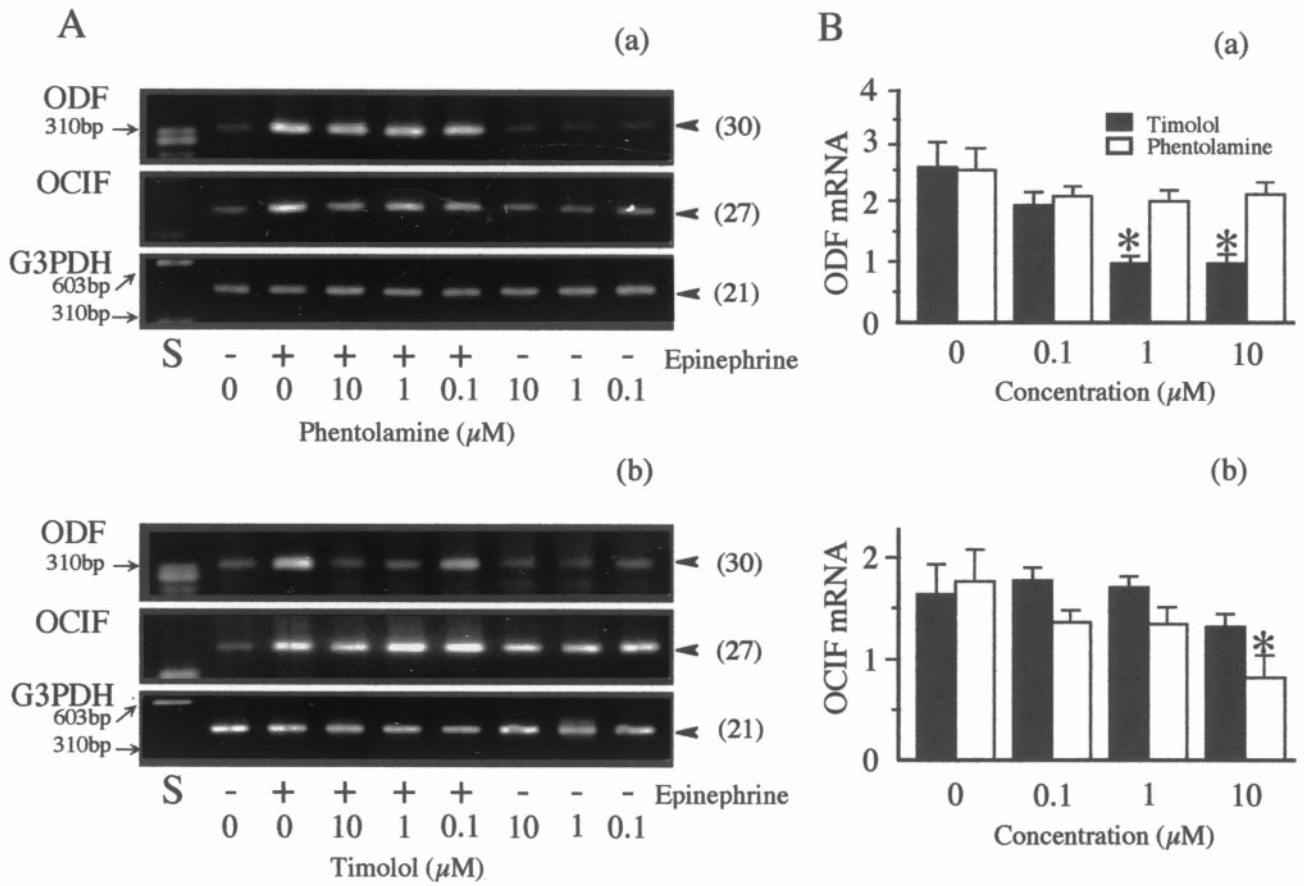

Fig. 4. Effects of phentolamine and timolol on induction of ODF and OCIF mRNAs in MC3T3-E1 cells treated with epinephrine. (A) RT–PCR analysis obtained from cells treated with epinephrine (1 μM) in the presence or absence of phentolamine (a) or timolol (b). The adrenergic antagonist was treated 30 min before the treatment with epinephrine. DNA size markers (Φ*X174/HaeIII* digest) are shown in the left lanes (S). Numbers in parentheses on the right indicate cycles of PCR amplification. Arrowheads indicate the predicted sizes of PCR products. (B) Relative expression of ODF (a) and OCIF (b) mRNAs in response to epinephrine (1 μM) in the presence or absence of phentolamine (0.1 to 10 μM) or timolol (0.1 to 10 μM). The mRNA levels of ODF and OCIF were calculated by dividing the intensity of the ODF and OCIF bands by that of the G3PDH band as determined by a fluorescence image analyzer. Pooled data from six independent experiments are expressed as means ± SEM (*$P < 0.05$, statistical difference from the value for no treatment with adrenergic antagonist, by Student's *t*-test).

rapid increase in ODF and OCIF mRNA levels, which peaked after 0.5 hr of treatment. IL-11 (10 ng/mL) caused a relatively delayed increase, peaking after 3 hr of treatment. The time-course experiment showed that the mRNA response to epinephrine was more rapid than that to IL-11.

To examine the involvement of PGE_2 in the action of epinephrine on the expression of ODF and OCIF mRNA, we tested the effect of NS-398, a well-known COX-II inhibitor, on the expression of ODF and OCIF mRNAs in MC3T3-E1 cells treated with epinephrine. As shown in Fig. 3A, the effect of epinephrine (0.1, 1, and 10 μM) on these mRNA levels was not influenced by pretreatment of the cells with NS-398 (100 μM). Since NS-398 completely inhibited the PGE_2 production resulting from treatment of cells with epinephrine (Fig. 3B), the data in panel A suggest that the heightened PGE_2 production in response to epinephrine did not stimulate the mRNA expression of ODF and OCIF.

Since it is well known that osteoblastic cells possess both α- and β-adrenergic receptors [11,12], the receptor involved in the mRNA expression of ODF and OCIF was determined pharmacologically (Fig. 4). The induction of ODF mRNA by epinephrine (1 μM) was inhibited by timolol, a β-antagonist, in a concentration-dependent manner (0.1 to 10 μM), but not by phentolamine (0.1 to 10 μM), an α-antagonist. On the other hand, induction of OCIF mRNA by epinephrine was inhibited by phentolamine only at the dose of 10 μM.

These findings led us to investigate the effects of epinephrine and isoproterenol on TRACP-positive MNC formation from mouse bone marrow cells. When mouse bone marrow cells were cultured for 7 days with epinephrine (10 μM) or isoproterenol (10 μM), TRACP-positive MNCs formed (Fig. 5A). No TRACP-positive MNCs appeared in the absence of these adrenergic agonists. As shown in Fig. 5B, epinephrine caused a slight but substantial stimulation of TRACP-positive MNC formation at a concentration of 0.1 to 10 μM. Furthermore, the formation of TRACP-positive MNCs stimulated by isoproterenol was much greater than that by

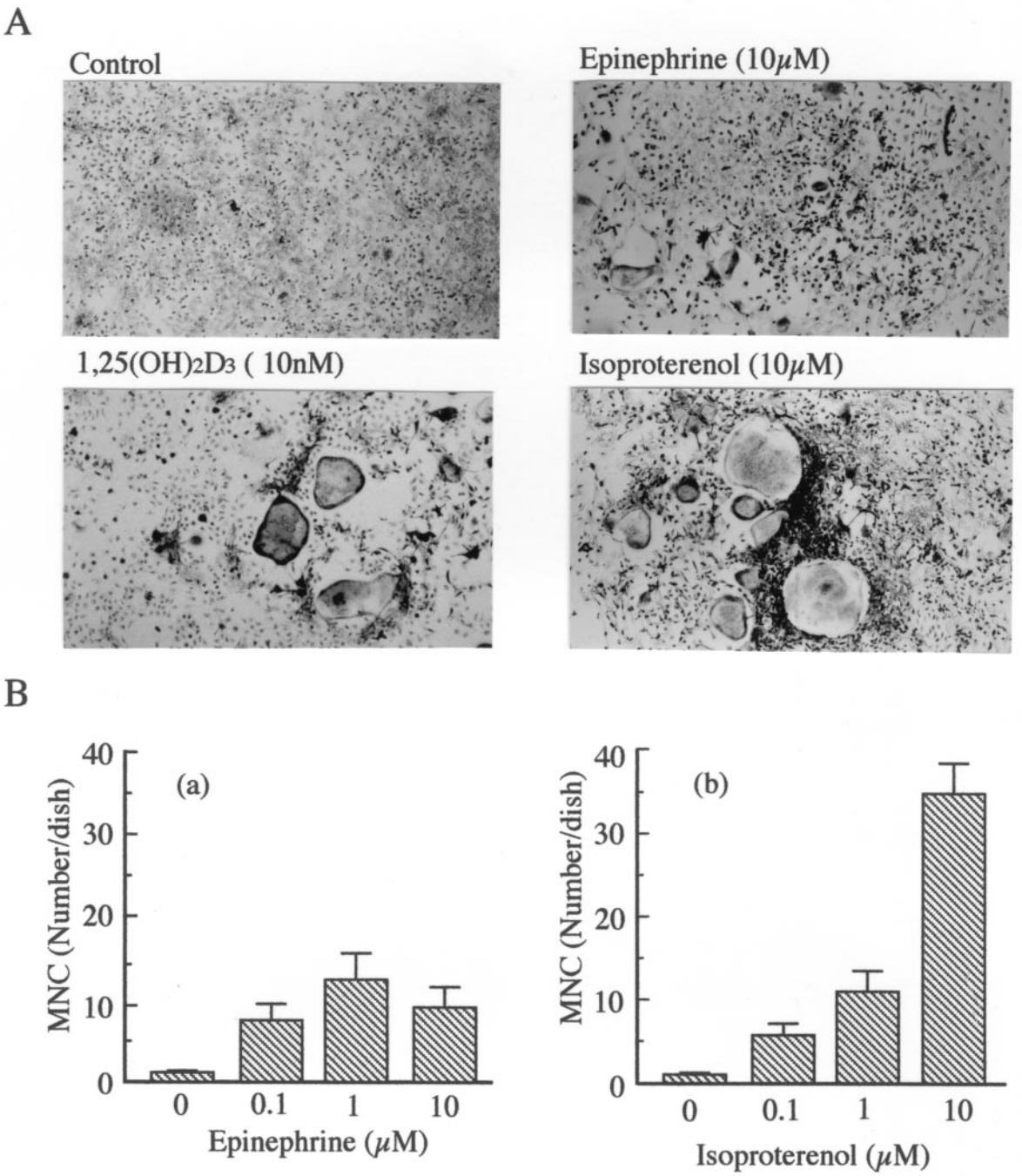

Fig. 5. Effect of epinephrine and isoproterenol on TRACP-positive MNC formation from mouse bone marrow cells. (A) Mouse marrow mononuclear cells were treated with vehicle, $1,25(OH)_2D_3$ (10 nM), epinephrine (10 μM), or isoproterenol (10 μM) for 7 days. The cells were then fixed and stained for TRACP-positive MNCs. $1,25(OH)_2D_3$ was used as positive control for the formation of TRACP-positive MNCs. (B) Mouse marrow mononuclear cells were treated with increasing concentrations of epinephrine (a) and isoproterenol (b). After cultures for 7 days, TRACP-positive MNCs were counted. Data are expressed as the means ± SEM of four cultures.

epinephrine and was dependent on the concentration added to the culture medium (0.1 to 10 μM).

4. Discussion

In the present study, for the first time increases in the mRNA expression of ODF and its decoy receptor, OCIF, were observed following treatment of MC3T3-E1 cells with epinephrine. An increase in ODF mRNA by IL-11, hPTH(1–34), or $1,25(OH)_2D_3$ in calvarial osteoblasts [23] and by $1,25(OH)_2D_3$ and dexamethasone in calvarial osteoblastic cells [24] was shown earlier. As for OCIF expression, the mRNA level of this protein was increased by IL-11, hPTH((1–34), or $1,25(OH)_2D_3$ in calvarial osteo-

blasts [23], by TNF-α or -β in osteosarcoma MG-63 cells [25], by IL-1α in osteosarcoma MG-63 cells [25], by IL-1β, TNF-α, or BMP (bone morphogenetic protein) -2 in a conditionally immortalized fetal human osteoblastic cell line (hFOB) [26], and by TGF-β1 in mouse primary osteoblasts [27]. On the other hand, there are other data showing a decrease in OCIF mRNA by $1,25(OH)_2D_3$ and dexamethasone in calvarial osteoblastic cells [24], and by $1,25(OH)_2D_3$, PGE_2, hPTH(1–34), or IL-1α in mouse primary osteoblasts [27]. Like these osteoclast-inducing factors, epinephrine showed a potency for increasing the mRNA expression of ODF and OCIF. However, the expression in response to epinephrine occurred in an immediate-early fashion, in comparison with the later expression elicited by the other factors.

It is well known that adrenergic agonists efficiently activate β-adrenoceptors on osteoblastic cells and that they can stimulate bone resorption in intact mouse calvaria. In neonatal mouse calvariae, adrenergic receptor agonists increased cAMP (cyclic adenosine monophosphate) production, while in the presence of a phosphodiesterase inhibitor and an antioxidant, they stimulated bone resorption [12]. Propranolol inhibited cAMP formation induced by β-adrenergic agonists in bone organ cultures [28] and increased bone strength and the rates of endochondral bone formation in rats [29]. In the present study, epinephrine showed the ability to elicit the production of osteotrophic factors such as IL-11 and PGE_2. In addition to these factors, ODF was also produced in response to epinephrine, indicating that the inducing action of epinephrine on ODF expression may not be based on a direct but rather an indirect effect of epinephrine via stimulation of IL-11 or PGE_2 production, which then could induce ODF mRNA. Because the mRNA expression of ODF occurred much faster in response to epinephrine than to IL-11 and was not influenced by the COX-II inhibitor, neither IL-11 nor PGE_2 was likely involved in the expression of ODF mRNA in response to epinephrine. Our data showing that IL-11 and PGE_2 were able to produce ODF in MC3T3-E1 cells indicate that ODF production was stimulated by the direct effect of epinephrine, as well as by its indirect effect via production of IL-11 and PGE_2.

The physiological action of epinephrine has recently been demonstrated to be mediated by β- as well as α-receptors. Suzuki *et al*. [11] demonstrated that epinephrine enhanced replication and ALP (alkaline phosphatase) activity of MC3T3-E1 osteoblastic cells via α1-adrenergic receptors coupled to Gi proteins, and concluded that PTX (pertussis toxin)-sensitive G proteins were potent mediators of cell proliferation and ALP activity of osteoblast-like cells in response to factors acting through G protein-coupled receptors. Our pharmacological results showed that different adrenergic receptors mediated ODF and OCIF expression: the expression of ODF and its decoy receptor, OCIF, was shown to be regulated by β- and α-adrenergic receptors, respectively. This led us to examine the effect of adrenergic agonists on osteoclastogenesis. The formation of osteoclast-like cells from mouse bone marrow cells was significantly increased by treatment with isoproterenol, but only slightly with epinephrine. The weak effect of epinephrine may be explained by the potency of epinephrine to produce not only ODF but also its decoy receptor, OCIF, in osteoblastic cells.

In conclusion, these findings suggest that expression of ODF and OCIF mRNA in osteoblastic cells may be regulated by adrenergic stimulation, without mediation of IL-11 and PGE_2 produced by epinephrine. The expression of ODF and OCIF by epinephrine appears to be mediated by β- and α-adrenergic stimulation, respectively. Since the osteoclastogenesis in mouse bone marrow cells was stimulated significantly more by isoproterenol than by epinephrine, osteoclastogenesis may be regulated by the balance between ODF and OCIF production in osteoblasts. At this time, the exact relationship between α- and β-adrenergic stimulation in osteoclastogenesis remains to be established.

Acknowledgments

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