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RECOMBINANT HUMAN INTERLEUKIN 1 ALPHA AND BETA STIMULATE MOUSE OSTEOBLAST-LIKE CELLS (MC3T3-E1) TO PRODUCE MACROPHAGE-COLONY STIMULATING ACTIVITY AND PROSTAGLANDIN E_2

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The effects of interleukin 1 (IL-1) on MC3T3-E1 cells (clonal osteoblast-like cells established from mouse calvaria) were studied to elucidate the mechanism of IL-1-induced bone resorption. Recombinant human interleukin 1 alpha (rhIL-1α) and beta (rhIL-1ß) stimulated PGE_2 production in MC3T3-E1 cells in a dose dependent manner. rhIL-1α and 1ß also stimulated MC3T3-E1 cells to produce macrophage-colony stimulating activity (M-CSA) in a dose-dependent manner. Indomethacin completely abolished PGE_2 production but did not affect CSA. These results suggest that bone resorption induced by IL-1s is at least in part mediated by PGE_2 produced by osteoblasts, and that M-CSA produced by osteoblasts may synergistically potentiate bone resorption by recruiting osteoclast precursors.

Interleukin 1 has a variety of biological effects such as T cell activation, prostaglandin E_2 (PGE_2) production in synovial fibroblasts and bone resorption (1). Indeed, IL-1 is the most potent bone resorbing factor in organ culture (2). Recently, osteoclast-activating factor (OAF) was proved to be identical to IL-1 beta (IL-1ß) (3). We previously reported that IL-1 alpha (IL-1α), which shares a number of the biological effects of IL-1ß, stimulated PGE_2 production and bone resorption in the forearm bones of fetal mice (4). This is also the case in IL-1ß-induced bone resorption (unpublished observation). To elucidate the mechanism of IL-1-induced bone resorption in fetal mouse bones, we studied the effects of IL-1 on MC3T3-E1 cells, a clonal osteoblast-like cell line derived from mouse calvaria (5), which retain many

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osteoblast-like features such as high alkaline phosphatase activity, receptors for 1,25-dihydroxyvitamin D_3 and mineralization in vitro (6). Very recently, this cell line was found to constitutively produce colony-stimulating activity (CSA) (7).

Materials and Methods

MC3T3-E1 cells were supplied from Dr. H. Kodama (Tohoku Dental University, Koriyama, Japan). MC3T3-E1 cells were cultured with MEM medium supplemented with 10 % fetal calf serum, penicillin (100 U/ml) and streptomycin (100 µg/ml) [MEM•FCS medium].

Recombinant human interleukin 1 alpha (rhIL-1α) with LAF activity of 2×10^7 U/mg of protein (8) and rhIL-1ß (LAF activity: 2×10^7 U/mg of protein) (9,10) were provided by Dainippon Pharmaceutical Co. (Suita, Osaka, Japan) and Otsuka Pharmaceutical Co. (Tokushima, Japan), respectively. Cells were cultured in 35mm-dishes with 2 ml MEM•FCS medium containing various concentrations of rhIL-1s, and the conditioned medium cultured for 3 days was kept at -20°C until assayed for CSA. In some experiments, 3×10^4 cells were plated into multidish 24 wells ($2cm^2$, Nunclon, Denmark). At the logarithmic phase and after confluency, cell monolayer was washed and the medium was replaced with 1 ml MEM medium containing 0.2 % crystalized bovine serum albumin (BSA, Miles Laboratories, IL) and various concentrations of rhIL-1s. After 2 days of culture, the conditioned medium was kept in plastic tubes at -20°C until assayed for PGE_2.

PGE_2 concentrations in the spent medium were determined by a radioimmunoassay kit (NEK-020, New England Nuclear Research Products, Boston, MA). The assay kit uses ^{125}I-labelled PGE_2 derivative as a tracer. The antibody used has the following cross-reactivity when calculated at the 50 % B/Bo point: PGE_2:100 %, PGE_1:3.7 %, PGA_1, PGB_2 and PGD_2:0.001 %.

CSA was assessed as described previously (11). In brief, 2 ml of 2.2 % methyl-cellulose (Methocel powder, 4000 cps, Dow Chemical Co., Midland, MI) in MEM medium, 1 ml horse serum (Gibco Laboratories, Grand Island, NY), 0.1 - 0.5 ml of conditioned medium, 0.9 - 0.5 ml MEM medium and 3×10^5 mouse bone marrow cells suspended in 1 ml MEM medium (total volume: 5.0 ml) were mixed well, and 1 ml of the mixture was plated in three tissue culture dishes (35 × 10 mm, Falcon Labware, Oxnard, CA). Incubation was carried out at 37°C in 5 % CO_2 and 95 % air. On day 7, the number of colonies (50 cells or more) was counted using an inverted microscope, and CSA was expressed by numbers of colonies formed per 60,000 bone marrow cells. Differences between colony counts were considered significant if the P value was < 0.05 by Student's t test.

Colonies were smeared onto glass slides and stained with May-Giemsa. To demonstrate the morphological type of colonies more clearly, they were also examined after double-staining for alpha-naphtol AS-D chloroacetate esterase and alpha-naphthyl butylate esterase (12).

To study rhIL-1 effects on cell growth, 2×10^4 cells were plated into multiwell dishes ($2\ cm^2$). On the following day, the medium was replaced with 1 ml MEM•FCS medium containing various concentrations of rhIL-1 α or ß, and the number of cells were counted every second day. MC3T3 cells proliferated with a doubling time of about 28 h and reached confluency on day 6. The number of cells increased to $3.4 \pm 0.15 \times 10^5$ /dish on day 8 in the absence of rhIL-1. rhIL-1s only slightly decreased the cell number [$3.1 \pm 0.19 \times 10^5$ (mean ± SD, n=4)] at 10 ng/ml on day 8.

Results

rhIL-1 effects on PGE_2 production: Similar to rat osteoblast-like cells (ROS 17/2 cells)(13), MC3T3-E1 cells produced and secreted PGE_2 in the conditioned

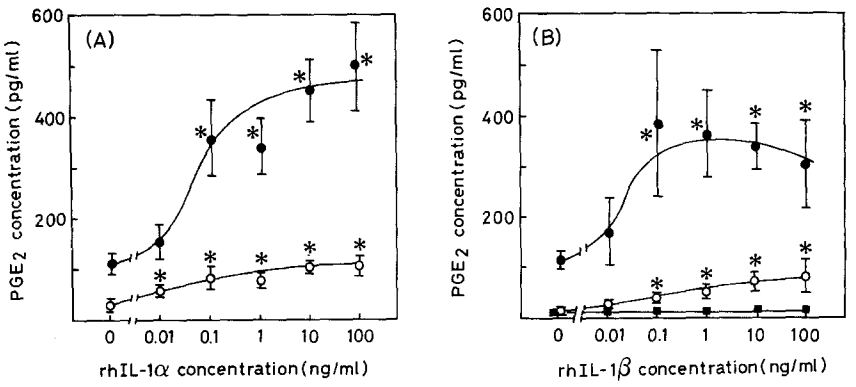

Figure 1. rhIL-1 effects on PGE_2 production in MC3T3-E1 cells

MC3T3-E1 cells (3×10^4 cells in 1 ml of MEM·FCS medium) were plated into 24-multiwell dishes ($2cm^2$) and the medium was changed every 3 days. On day 5 of the logarithmic phase and on day 11 of the over-confluent phase, the medium was replaced with 1 ml MEM medium containing 0.2 % BSA and various concentrations of rhIL-1 in the presence or absence of indomethacin (2×10^{-6} M). After 2 days of culture, the conditioned medium was stored, and PGE_2 concentrations were determined by RIA kit. Data are the mean ± SD of quadruplicate cultures. Abscissa indicates rhIL-1 α (left panel) and rhIL-1ß (right panel) added to the culture medium.
○—○: Conditioned medium on day 5-6 without indomethacin.
●—●: Conditioned medium on day 11-12 without indomethacin.
■—■: Conditioned medium on day 11-12 with indomethacin.

medium. As shown in Fig.1, rhIL-1 α and ß stimulated mouse osteoblast-like cells to produce PGE_2 in a dose-dependent manner. The minimal IL-1α and ß concentrations necessary to elicit significant PGE_2 production were observed at 10 - 100 pg/ml, and this stimulatory effect was more marked in the over-confluent phase than in the logarithmic phase. The addition of indomethacin to the culture medium completely abolished basal as well as IL-1-stimulated PGE_2 production.

rhIL-1 effects on CSA produced by MC3T3-E1 cells: MC3T3-E1 cells produced CSA (Fig.2), which is more potent in the conditioned medium obtained in the over-confluent phase than in the logarithmic phase. Both rhIL-1α and rhIL-1ß potentiated CSA in the conditioned medium in a dose-dependent manner. The stimulatory effect was significantly detected at 100 pg/ml. Nonconditioned medium containing the same IL-1 concentrations produced no colony formation. Furthermore, the addition of anti-IL-1 antiserum (which completely inactivated thymocyte proliferation) just prior to the CSA bioassay did not abolish the

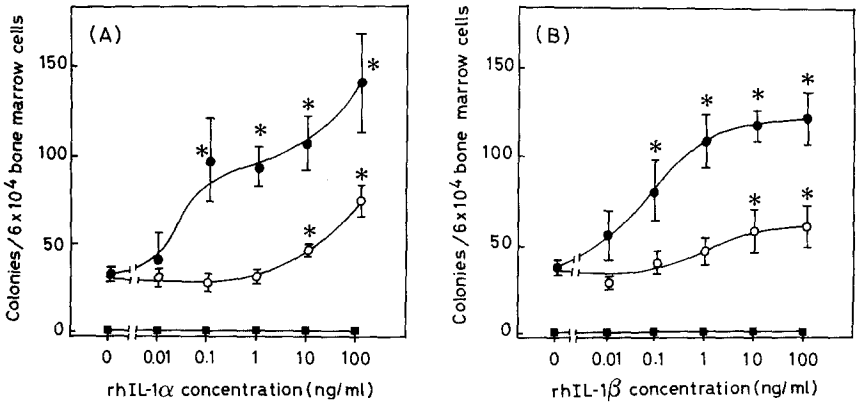

Figure 2. rhIL-1 effects on CSA production by MC3T3-E1 cells

MC3T3-E1 cells (10^5 cells in 2 ml MEM•FCS medium) were plated into 35 mm dishes. On the following day, the medium was replaced with 2 ml MEM•FCS medium containing various concentrations of rhIL-1. The conditioned medium obtained in the over-confluent phase (on day 8-11; 1×10^6 cells/dish) was stored. CSA was assessed as described in the Materials and Methods, using 0.1 ml (2 %) of the conditioned medium (●—●). Nonconditioned medium containing the same concentrations of rhIL-1s produced no colony formation (■—■). To study IL-1 effects on mouse bone marrow cells in the presence of CSA, rhIL-1 was added to the conditioned medium cultured without rhIL-1 just prior to CSA assay (○—○). The data indicate that rhIL-1 has weak but significant CSA-potentiating effects at a final concentration of 0.2-2 ng/ml in the CSA bioassay mixture. Data are the mean ± SD of triplicate cultures. Abscissa indicates rhIL-1α (left panel) and rhIL-1ß (right panel) added to the MEM•FCS medium. All assays were performed using 0.1 ml (2 %) of the conditioned or nonconditioned medium. * $p < 0.05$, compared with the conditioned medium without rhIL-1.

IL-1-induced CSA of the MC3T3-E1-conditioned medium (Table 1), suggesting that IL-1s directly stimulated osteoblast-like cells to produce CSA. However, when rhIL-1s were added to the conditioned medium of MC3T3-E1 cells cultured without IL-1 just prior to the CSA bioassay, a slight but significant increase in the number of colonies was observed at an IL-1 concentration of 10 - 100 ng/ml. Since 0.1 ml (2 %) of the conditioned medium was used for CSA bioassay, these data suggest that rhIL-1s are also capable of potentiating the osteoblast-derived CSA in the mouse bone marrow cells at a final concentration of 0.2 - 2 ng/ml in the CSA bioassay. In contrast to PGE_2 production, the addition of indomethacin into the MEM•FCS medium did not affect either basal or IL-1-stimulated CSA produced by MC3T3-E1 cells (data not shown).

Characterization of colonies stimulated by MC3T3-E1 cell CSA: After 7 days of culture, all the colonies were composed of alpha-naphthyl butylate-positive

Table 1. anti-rhIL-1 antiserum effect on rhIL-1-induced CSA of MC3T3-E1 cells

MC3T3-E1 conditioned medium	Colonies/6 × 10^4 bone marrow cells	
	Antiserum (-)	Antiserum (+)
rhIL-1α (-)	20.1 ± 4.5	18.3 ± 2.0
10 pg/ml	31.0 ± 9.1	26.7 ± 7.7
100 pg/ml	67.3 ± 7.4	75.3 ± 10.2
1,000 pg/ml	65.6 ± 9.3	78.0 ± 15.7

The conditioned medium of MC3T3-E1 cells cultured with various concentrations of rhIL-1α (10-1000 pg/ml) for 3 days was assayed for CSA as described in Materials and Methods. Polyclonal anti-rhIL-1α antiserum, which completely inactivated IL-1 activity in the conditioned medium (4), was added to the CSA bioassay. Data are the number of colonies formed per 6 × 10^5 mouse bone marrow cells after 7 days of culture. Note that anti-IL-1 antiserum does not affect the IL-1α-induced CSA in the MC3T3-E1-conditioned medium.

macrophages. Neither alpha-naphtol AS-D chloroacetate-positive granulocyte colonies nor granulocyte-macrophage colonies were found. This was also true of the IL-1-potentiated colonies. These results suggest that MC3T3-E1 cells produce predominantly macrophage-colony stimulating activity (M-CSA).

Discussion

rhIL-1α stimulates bone resorption in the forearm bones of fetal mice by PGE_2-dependent as well as by PGE_2-independent mechanisms (4). The radii and ulnae of fetal mouse bones used for bone resorption bioassays (4,11) are composed of various types of cells such as fibroblasts, osteogenic cells like osteocytes and osteoblasts, endothelial cells, osteoclasts and hematopoietic cells. The present results suggest that both rhIL-1α and rhIL-1ß stimulate osteoblasts in fetal bones to produce PGE_2. Since human monocyte-derived IL-1 can also stimulate fibroblasts to produce PGE_2 (14), we have speculated that IL-1 stimulates both osteoblasts and fibroblasts to produce PGE_2, which directly (15) or indirectly (16) enhances osteoclastic bone resorption in fetal mouse bones. It remains to be elucidated whether or not other cells such as endothelial cells and bone marrow cells respond to IL-1 and produce PGE_2.

CSA is produced constitutively by a number of transformed or nontransformed cell lines (17). We have found that MC3T3-E1 cells produce predominantly macrophage-colony stimulating factor (M-CSF) like mouse L-cells

(17) and that the production of M-CSA by the osteoblast-like cells was stimulated by IL-1 as it stimulates fibroblasts to produce GM-CSA (14).

In summary, we have demonstrated that mouse osteoblast-like cells produced and released PGE_2 in a similar fashion to rat osteoblast-like cells (ROS 17/2)(13), and that rhIL-1 stimulated the production of PGE_2 in MC3T3-E1 cells as PTH did in human osteoblasts (18). Since PGE_2 is a potent stimulator of bone resorption (15), our findings indicate a possible osteoblastic contribution to IL-1-stimulated osteoclastic bone resorption (19). Furthermore, since osteoclasts are believed to derive from the macrophage/monocyte series, it is possible that GM-CSF and/or M-CSF produced by fibroblasts and osteoblasts may potentiate osteoclastic bone resorption, presumably by recruiting osteoclast precursors in fetal mouse bones (11).

Acknowledgments

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