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TUMOR NECROSIS FACTOR TYPE α (CACHECTIN) STIMULATES MOUSE OSTEOBLAST-LIKE CELLS (MC3T3-E1) TO PRODUCE MACROPHAGE-COLONY STIMULATING ACTIVITY AND PROSTAGLANDIN E_2

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To elucidate the mechanism of tumor necrosis factor alpha (TNF-α)-induced bone resorption, the effects of recombinant human TNF-α on mouse osteoblast-like cells (MC3T3-E1) were studied. TNF-α stimulated MC3T3-E1 cells to produce prostaglandin E_2 (PGE_2) and macrophage colony stimulating activity (M-CSA) in a dose-dependent manner. TNF decreased alkaline phosphatase (AL-P) activity of MC3T3-E1 cells. These TNF effects were observed at 1 ng/ml (~6×10^{-11}M). The inhibitory effect on AL-P activity was reversible and the cell growth of MC3T3-E1 cells was only slightly affected by TNF. These findings suggest that both PGE_2 and M-CSA stimulated by TNF-α are possibly involved in osteoblast-mediated osteoclastic bone resorption, whereas inhibition of AL-P activity may lead to a decrease in bone formation.

Tumor necrosis factor alpha (TNF-α) or cachectin (1) is a potent bone resorbing factor, which substantially accounts for the bone resorbing activity of osteoclast-activating factors (OAFs) produced by peripheral blood leukocytes (2,3). Previously we have demonstrated that recombinant human interleukin 1 alpha (IL-1α), another OAF candidate, stimulates bone resorption probably via an osteoblast-mediated mechanism (4,5). Since TNF/cachectin and IL-1 share a number of biological activities (1,6), we investigated the effects of TNF on osteoblasts using mouse osteoblast-like cells (MC3T3-E1), which retain several osteoblastic features, such as receptors for PTH and 1,25-dihydroxyvitamin D_3, calcification in vitro, and high alkaline phosphatase (AL-P) activity (7,8).

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Materials and Methods

MC3T3-E1 cells were supplied by Dr. Kodama (Tohoku Dental University, Koriyama, Japan)(7). The clonal cells were cultured in MEM medium supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 µg/ml) (MEM/FCS medium). Recombinant human TNF-α (PT-050, 2.9×10^6U/mg protein) was supplied by Dainippon Pharmaceutical Co. (Suita, Osaka, Japan) (9). Cells were cultured in 35-mm dishes with 2 ml of MEM/FCS medium containing various concentrations of TNF. The conditioned medium cultured for 3 days was kept at -20°C until assayed for colony-stimulating activity (CSA) and PGE_2 concentration.

In order to study the effects of TNF on cell growth and AL-P activity, 5×10^4 MC3T3-E1 cells in 2 ml of MEM/FCS medium were plated into 35-mm dishes. On the following day, the medium was replaced with MEM/FCS medium containing various concentrations of TNF-α, unless otherwise specified. After an appropriate number of days in culture, spent medium was aspirated and monolayer cells were trypsinized. The numbers of cells were then counted by Coulter cell counter.

When AL-P activity was studied, monolayer cells were rinsed with Hanks' solution, scraped into 0.2% Nonidet P-40 containing 1 mM $MgCl_2$(8), and sonicated with a sonicator (Microson, Ultrasonic Cell Disruptor, Farmingdale, N.Y.) for 10 sec. AL-P activity was determined according to the method of Lowry (10). One unit of enzyme activity equals 1 µmol product (paranitrophenol) formed per min. Proteins were measured by the method of Bradford (11), using bovine serum albumin as a standard.

CSA was assessed as described previously (12). In brief, 2 ml of 2.2% methylcellulose (Methocel powder, 4000 cps, Dow Chemical Co., Midland, MI) in MEM medium, 1 ml horse serum (Gibco Laboratories, Grand Island, N.Y.), 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 ml) were mixed well, and 1 ml of mixture was plated in 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 numbers of colonies (50 cells or more) were counted using an inverted microscope, and CSA was expressed as the number of colonies formed per 60,000 bone marrow cells. Differences between colony counts were considered significant if the P values were < 0.05 by Student's t test.

PGE_2 concentrations in the spent medium were determined with a radioimmunoassay kit (NEK-020A, NEN Research Products, N. Billerica, MA). Samples were acidified to pH 3.5 and purified through Bond-Elute C-18 extraction columns (Analytichem International, Harbor City, CA) exactly as described in the brochure issued by the supplier. When [^{3}H]PGE_2 was added to the samples, more than 95% of radioactivity was recovered. The antibody has the following cross-reactivities when calculated at the 50% B/Bo point: PGE_2:100%, PGE_1:3.7%, PGF_1:0.03% and arachidonic acid:<<0.01.

Results

1) Effects of TNF-α on cell growth and AL-P activity of MC3T3-E1 cells: As shown in Fig.1A, MC3T3-E1 cell growth was dependent on FCS concentration. TNF-α did not affect cell growth for the first 5 days of culture and had only slightly inhibited it by 7 days of culture. Basal AL-P activity increased steadily, especially after the cells had reached confluency (~1×10^6 cells/35-mm dish or $2\sim3\times10^5$/24-multiwell dish/2 cm^2). In contrast to these minimal effects on cell growth, TNFα greatly decreased AL-P activity in mouse osteoblast-like cells in a concentration-dependent manner (Table 1). One ng/ml TNF-α significantly

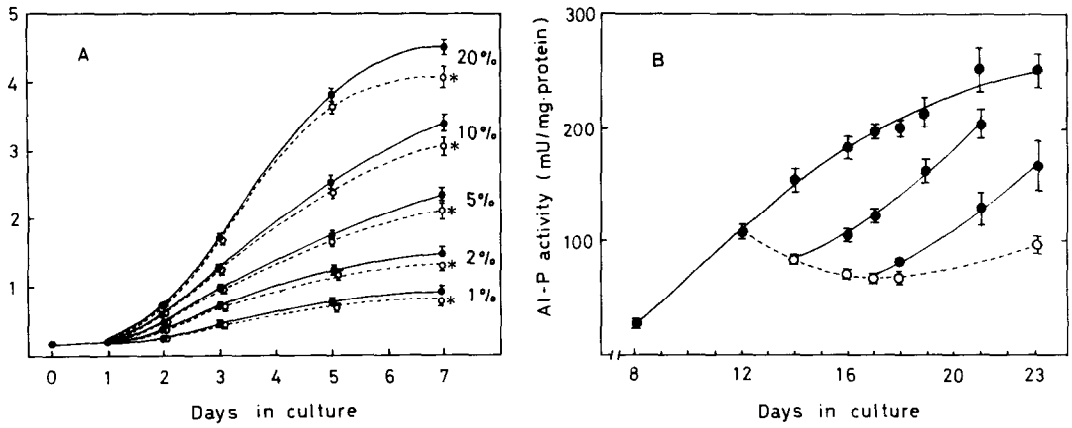

Fig.1. Effects of TNF-α on cell growth (left) and AL-P activity (right)

(A) MC3T3-E1 cells (2×10^4/1 ml MEM/FCS medium) were plated into 24 multiwell plates (2 cm^2/dish). On the following day, medium was replaced with 1 ml MEM medium containing various concentrations of FCS with (○-○) or without (●—●) TNF-α (50 ng/ml). At 2, 3, 5 and 7 days of culture, numbers of cells were counted. When cells were cultured in MEM/FCS medium (10% FCS), cells reached confluency 5 days after inoculation ($2\sim3\times10^5$/2 cm^2). Data are means ± SD of triplicate cultures. Where error bars are not shown, SD is less than the diameter of the circles. * $p < 0.05$.
(B) MC3T3-E1 cells (5×10^4/2 ml MEM/FCS medium) were plated into 35-mm dishes and cultured for 12 days, then medium was replaced with MEM/FCS medium containing 40 ng/ml TNF-α (○-○). After an additional 2 or 5 days of culture, some of these cells were cultured again with MEM/FCS medium (●—●). Data are means ± SD of triplicate cultures.

decreased the enzyme activity (Table 1). This inhibitory effect on AL-P activity was reversible when cells were cultured in TNF-free MEM/FCS medium (Fig.1B).

Table 1. Effects of TNF-α on cell growth and AL-P activity of MC3T3-E1 cells

TNF-α concentration (ng/ml)	Cell number ($\times10^{-6}$/dish)	AL-P activity (mU/mg protein)	
	day 9	day 9	day 13
0	1.14 ± 0.02	18.6 ± 2.0	65.8 ± 2.1
0.01	1.17 ± 0.08	20.7 ± 2.9	66.0 ± 1.9
0.1	1.11 ± 0.03	18.6 ± 0.9	67.8 ± 4.0
1.0	1.10 ± 0.02	13.6 ± 0.7*	58.9 ± 8.7*
10	1.16 ± 0.03	6.3 ± 0.3**	36.0 ± 1.1**
100	1.07 ± 0.02*	4.5 ± 0.6**	10.6 ± 0.2**

MC3T3-E1 (5×10^4) cells were plated into 35-mm dishes. On the following day, medium was replaced with 2 ml MEM/FCS medium containing various concentrations of TNF-α. Medium was changed twice a week. On the 9th day of culture, cell numbers were counted and, on days 9 and 13, AL-P activity was determined as described in Methods. Data are means ± SD of triplicate cultures for cell number and quadruplicate cultures for AL-P activity. * $p < 0.05$, ** $p < 0.001$

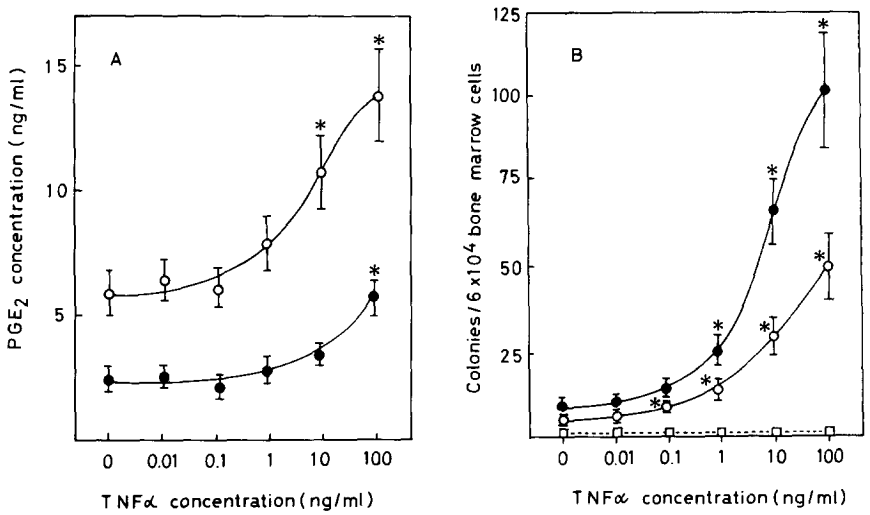

Fig.2. Effects of TNF-α on PGE_2 and CSA produced by MC3T3-E1 cells

MC3T3-E1 cells were plated into 35-mm dishes. When cells became subconfluent (~3×10^5 cells/dish) or superconfluent (~10×10^5 cells/dish), medium was replaced with 2 ml MEM/FCS medium and cultured for an additional three days. The conditioned medium was then assayed for PGE_2 (A) and CSA (B). Data are means ± SD of triplicate cultures. For CSA assay, 2% of the conditioned medium was used.
●—● Conditioned medium obtained in the superconfluent phase.
○—○ Conditioned medium obtained in the logarithmic phase.
□ - □ Nonconditioned medium containing TNF-α.

2) Effect of TNF-α on PGE_2 production in MC3T3-E1 cells: TNF-α stimulated MC3T3-E1 cells to produce PGE_2 in a concentration-dependent manner (Fig.2A and Table 2). Minimal effective dose was observed at 1 ng/ml (Table 2). Stimulation

Table 2. Effects of indomethacin on PGE_2 and CSA production by MC3T3-E1 cells

TNF-α concentration (ng/ml)	PGE_2 (ng/ml)		CSA (colonies/dish)	
	Ind(-)	Ind(+)	Ind(-)	Ind(+)
0	4.2 ± 0.2	0.20 ± 0.01	9.3 ± 1.2	7.6 ± 1.5#
0.01	3.8 ± 0.4	0.22 ± 0.03	11.6 ± 1.5	11.0 ± 5.2#
0.10	4.5 ± 0.5	0.20 ± 0.06	16.3 ± 4.9	16.6 ± 5.8#
1.0	6.0 ± 0.3*	0.19 ± 0.02	47.0 ± 10.5	36.0 ± 9.0#
10	7.9 ± 0.6*	0.22 ± 0.05	62.0 ± 2.6	58.8 ± 2.9#
100	8.4 ± 1.2*	0.19 ± 0.04	104.3 ± 13.4	94.0 ± 8.9#

MC3T3-E1 cells (5×10^4 cells/2 ml MEM/FCS medium) were plated into 35-mm dishes. When cells were exponentially growing, medium was replaced with fresh MEM/FCS medium containing various concentrations of TNF-α and indomethacin (10^{-6}M), and cells were cultured for an additional three days. Conditioned medium was assayed for CSA and PGE_2 as described in Methods. For CSA assay, 0.2 ml of the conditioned medium (4%) was used. All data are means ± SD of triplicate cultures. * $p < 0.01$, # $p > 0.1$, Ind(-) vs. Ind(+)

Table 3. Effect of TNF-α on colony formation by murine bone marrow cells

	TNF-α concentration (ng/ml)			
	0	1	10	100
Number of colonies	119 ± 10	121 ± 11	110 ± 4	92 ± 6*

Murine bone marrow cells (6×10^4 cells/dish) were cultured with 15% MC3T3-E1 conditioned medium obtained in the superconfluent phase. TNF-α was added, just prior to the bioassay, to a final concentration as described above. The data represent numbers of colonies formed after 7 days of culture (mean ± SD, n=3). Note that TNF-α is not so toxic to murine bone marrow cells as to human bone marrow cells in terms of macrophage colony formation (24).
* $p < 0.05$

of PGE_2 production was more marked in the logarithmic phase than in the superconfluent phase (Fig.2A). Addition of indomethacin to the culture medium almost completely abolished basal as well as TNF-induced PGE_2 production (Table 2).

3) Effect of TNF-α on CSA production in MC3T3-E1 cells: As reported previously, MC3T3-E1 cells constitutively produced CSA which predominantly stimulated macrophage colony formation (5). TNF-α also stimulated CSA production in a concentration-dependent manner (Fig.2B). As little as 0.1~1 ng/ml of TNF-α stimulated osteoblast-like cells to release CSA. No CSA was detected in nonconditioned MEM/FCS medium containing various concentrations of TNF-α (Fig.2B). Furthermore, when TNF-α was directly added to the culture mixture for CSA bioassay, it slightly inhibited macrophage colony formation induced by M-CSA of MC3T3-E1 cells at a concentration of only 100 ng/ml, suggesting that TNF-α directly stimulated MC3T3-E1 cells to produce CSA (Table 3). In contrast to PGE_2, TNF-α-stimulated CSA was not affected by indomethacin (Table 2).

Discussion

Generally AL-P is regarded as a marker enzyme for bone formation (13). We have demonstrated in non-transformed osteoblast-like cells that TNF inhibits AL-P activity, as has been reported for osteosarcoma cells (ROS 17/2.8)(2). The TNF effect on the enzyme activity was not due to a cytotoxic effect on MC3T3-E1 cells: TNF-α-inhibited AL-P activity was reversible and MC3T3-E1 cell growth was hardly affected, as has been reported for several murine cell lines (14).

Although TNF-α and IL-1 are different molecules and bind to different receptors (15), it is becoming evident that they share a number of biological

activities, such as production of fever, lipolysis, production of PGE_2 in various cells, and bone resorption (1,6). We have found that TNF-α, like IL-1α and ß, also stimulates mouse osteoblast-like cells to produce PGE_2 and M-CSA (5). These findings are comparable with recent reports that TNF-α stimulates endothelial cells and fibroblasts to produce granulocyte-macrophage CSF (GM-CSF) (16,17) and PGE_2 (18).

PGE_2 is a potent bone resorbing agent (19) and M-CSA is a growth factor which stimulates macrophage colony formation and may recruit osteoclast precursors. Since M-CSA is known to stimulate macrophages to produce IL-1 (20), it is possible that both cytokines are in some way involved in physiological bone remodelling or pathological bone resorption (21).

Tashjian et al. (22) have reported that TNF-α-stimulated bone resorption is mediated via prostaglandin synthesis and that indomethacin completely abolishes TNF-α effect in neonatal mouse calvaria. Our present data also suggest that PGE_2 produced by osteoblasts is probably involved in TNF-α-stimulated bone resorption. More recently, Chambers et al. (23) have reported that TNF-α-induced bone resorption is mediated by osteoblast products of low molecular weight which seems to be different from PGE_2. Although it is highly likely that these unidentified osteoblast products are responsible for TNF-mediated bone resorption, we speculate that PGE_2 and M-CSA produced by osteoblasts are at least partly involved in TNF-α-stimulated osteoclastic bone resorption, as with IL-1-induced bone resorption (4,5).

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