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Transient Expression of M-CSF Is Important for Osteoclast-Like Cell Differentiation in a Monocytic Leukemia Cell Line

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Abstract Cells of U937, a human monocytic leukemia cell line, differentiate into macrophages by treatment with 12-o-tetradecanoylphorbol-13-acetate (TPA), whereas cells treated with 1α,25-dihydroxyvitamin D3 [1,25-$(OH)_2D_3$] continue to grow without undergoing differentiation. When U937 cells were successively treated with TPA and 1,25-$(OH)_2D_3$, tartrate-resistant acid phosphatase-positive multinucleated cells appeared at 5 days after the treatment. These osteoclast-like cells released a soluble form of ^{45}Ca from ^{45}Ca-labeled bone particles. These cells were not formed when the order of treatment with TPA and 1,25-$(OH)_2D_3$ was reversed. Use of either dexamethasone or interferon-γ (IFN-γ) was effective in inhibiting the formation of these osteoclast-like cells. The expression of c-src, c-fms, and macrophage colony stimulating factor (M-CSF) was induced by TPA treatment; however, TPA-induced M-CSF gene transcription was attenuated by the subsequent addition of 1,25-$(OH)_2D_3$. Furthermore, both dexamethasone and IFN-γ impaired the attenuation of M-CSF expression, suggesting that the transient expression of M-CSF may be important for the formation of osteoclast-like cells. J. Cell. Biochem. 64:67–76. © 1997 Wiley-Liss, Inc.

Key words: TRAP; bone resorption; M-CSF; c-fms; monocytic cell

Osteoclasts and macrophages are derived from a common hemopoietic progenitor cell. Mixed bone marrow cells [Burger et al., 1984; Fuller and Chambers, 1987; Takahashi et al., 1988; Kukita et al., 1992], monocytes of peripheral blood [Quinn et al., 1994], and alveolar macrophages [Abe et al., 1983], were all shown to be capable of differentiating into osteoclasts or osteoclast-like cells. 1α,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$], which is a strong promoter of osteoclast development and is known to stimulate bone resorption, and unknown factor(s) derived from stromal cells are necessary for inducing fusion of precursor cells to form multinucleated cells resembling osteoclasts. The precise mechanisms of the normal differentiation processes of osteoclast formation, however, have not been fully elucidated because of the difficulty in refining the analysis system.

To elucidate the biochemical processes of osteoclast differentiation, cell lines derived from leukemia have been used as experimental material [Bar-Shavit et al., 1983; Biskobing and Rubin, 1993; Gattei et al., 1992; Benayahu et al., 1994]. A mouse myeloblastic cell line, M1, differentiated into osteoclastic cells as a result of coculture with marrow stomal adipocytes [Benayahu et al., 1994]. Cells of a human promyelocytic leukemia cell line, HL-60, were induced to differentiate into osteoclast-like cells by treatment with 1,25-$(OH)_2D_3$ [Biskobing and Rubin, 1993]. Recently, cells of another human leukemic cell line, FLG29.1, were reported to differentiate into osteoclast-like cells by treatment with 12-o-tetradecanoylphorbol-13-acetate (TPA) [Gattei et al., 1992]. The human monocytic leukemic cell line, U937, is made up of a very homogeneous cell population in which almost all cells can differentiate into macrophages as a result of treatment with TPA [Hosoya and Marunouchi, 1992]. However, neither 1,25-$(OH)_2D_3$ nor TPA were able to induce U937

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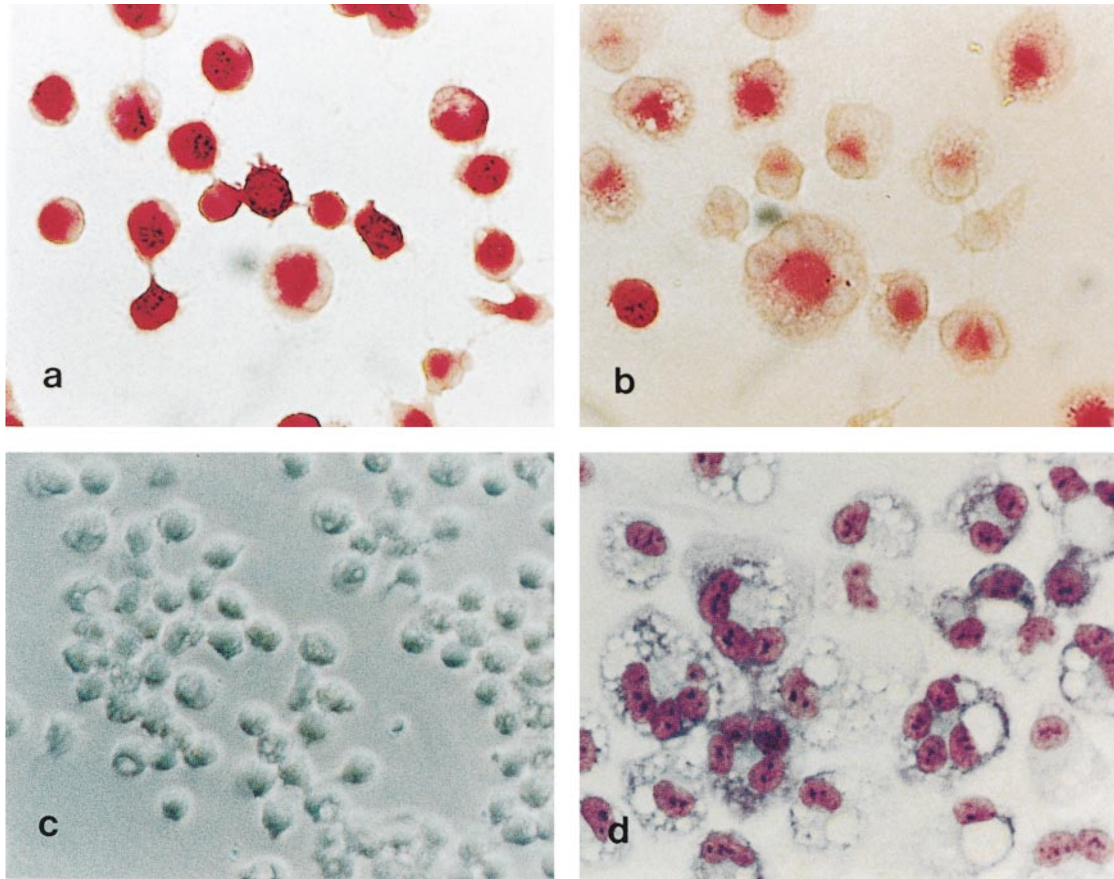


Fig. 1. Formation of TRAP-positive multinucleated cells (TPMCs). U937 cells were treated with TPA for 2 days **(c)**, incubated in the absence **(a)** or presence **(b** and **d)** of 1,25-$(OH)_2D_3$ for 7 days, fixed and stained for TRAP (a, b, and c) or by Giemsa (d).

to differentiate into osteoclast-like cells when used alone. Since we found that sequential treatment of U937 with TPA and 1,25-$(OH)_2D_3$ induced these cells to differentiate into osteoclast-like cells, we examined changes in phenotypes and gene expression after treatment with these reagents. TPA treatment resulted in the expression of c-fms, the macrophage colony stimulating factor (M-CSF) gene, and c-src, and subsequent treatment with 1,25-$(OH)_2D_3$ attenuated M-CSF expression, suggesting that transient M-CSF expression was closely correlated with osteoclast-like cell formation.

MATERIALS AND METHODS

Materials

12-o-tetradecanoyl-phorbol-13-acetate (TPA), 4α-phorbol-12,13-didecanoate, mezerein, dexamethasone, insulin, naphthol AS-MX phosphate, fast red violet LB salt, and an α-naphtyl acetate esterase kit were purchased from Sigma (St. Louis, MO). Other reagents were purchased from the following companies. 1α,25-dihydroxyvitamin D3 [1,25-$(OH)_2D_3$] was from Biomolecular Research Lab Inc. (Plymouth Meeting, PA), H7 [1-(5-isoquinolinyl-sulfonyl)2-methyl-piperazine] was from Research Biochem Inc. (Natick, MA), parathyroid hormone was from Peninsula Lab Inc. (Belmont, CA), human interferon-γ (IFN-γ) was from Toray (Tokyo, Japan), Taq DNA polymerase and RNase inhibitor were from Promega (Madison, WI), M-MLV reverse transcriptase and random primer were from Life Technologies Inc. (Gaithersburg, MD), hepatocyte growth factor, culture dishes and multitestplates were from Becton Dickinson Lab (Lincoln Park, NJ), and fetal calf serum was from GIBCO (Grand Island, NY).

Cell Culture

U937, a human monocytic leukemia cell line, and ST2, a mouse stromal cell line, were cultured in RPMI 1640 medium supplemented with

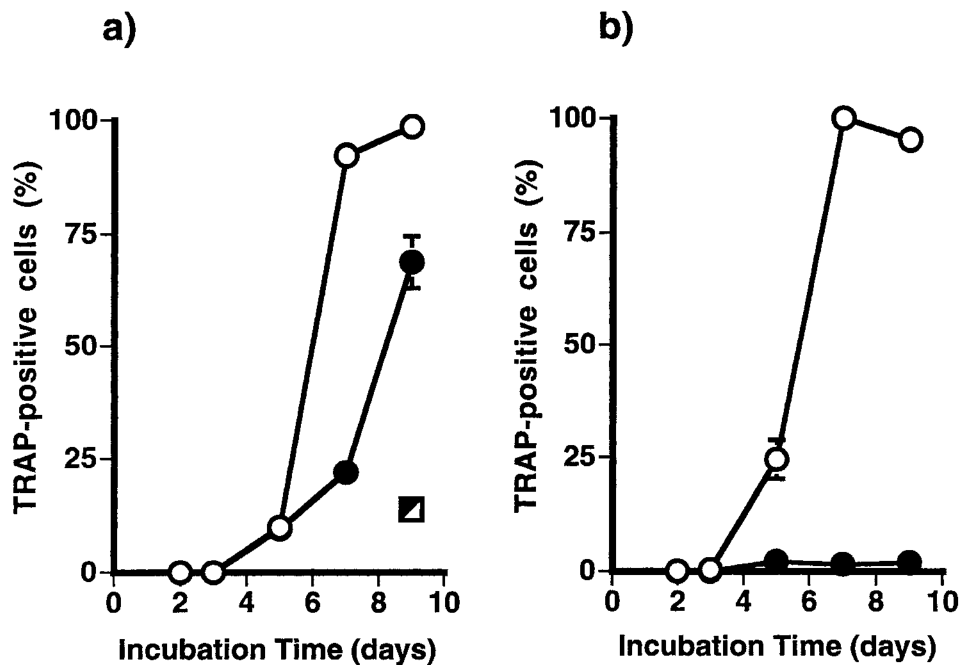


Fig. 2. Time course of TPMC formation. U937 cells treated with TPA were incubated with (a) or without (b) 1,25-$(OH)_2D_3$ for 7 days. Samples were taken, fixed, and stained for TRAP at the indicated times. TRAP-positive cells (○) or TPMCs (●) were counted. ◪, TPMC formation in the continuous presence of TPA. Bars = S.E. for quadruplicate assays.

10% fetal calf serum at 37°C in a humidified atmosphere of 95% air, 5% CO_2.

The standard procedure for inducing differentiation of U937 cells was as follows. Exponentially growing cells were harvested and suspended in medium containing 0.1 μg/ml TPA at a concentration of 2×10^5 cells/ml. Then, 100 μl of the suspension were seeded in wells of 24-well multitestplates, each of which contained a cover glass (d = 14 mm), and incubated at 37°C for 48 h. After treatment with TPA, differentiated adherent cells were washed twice with calcium magnesium-free phosphate buffered saline (PBS), treated with medium containing 10^{-8} M 1,25-$(OH)_2D_3$, and incubated for 7 days.

The number of nuclei was counted after cell staining in more than 400 cells in each of 4 samples. Cells containing two or more nuclei were judged to be multinuclear cells.

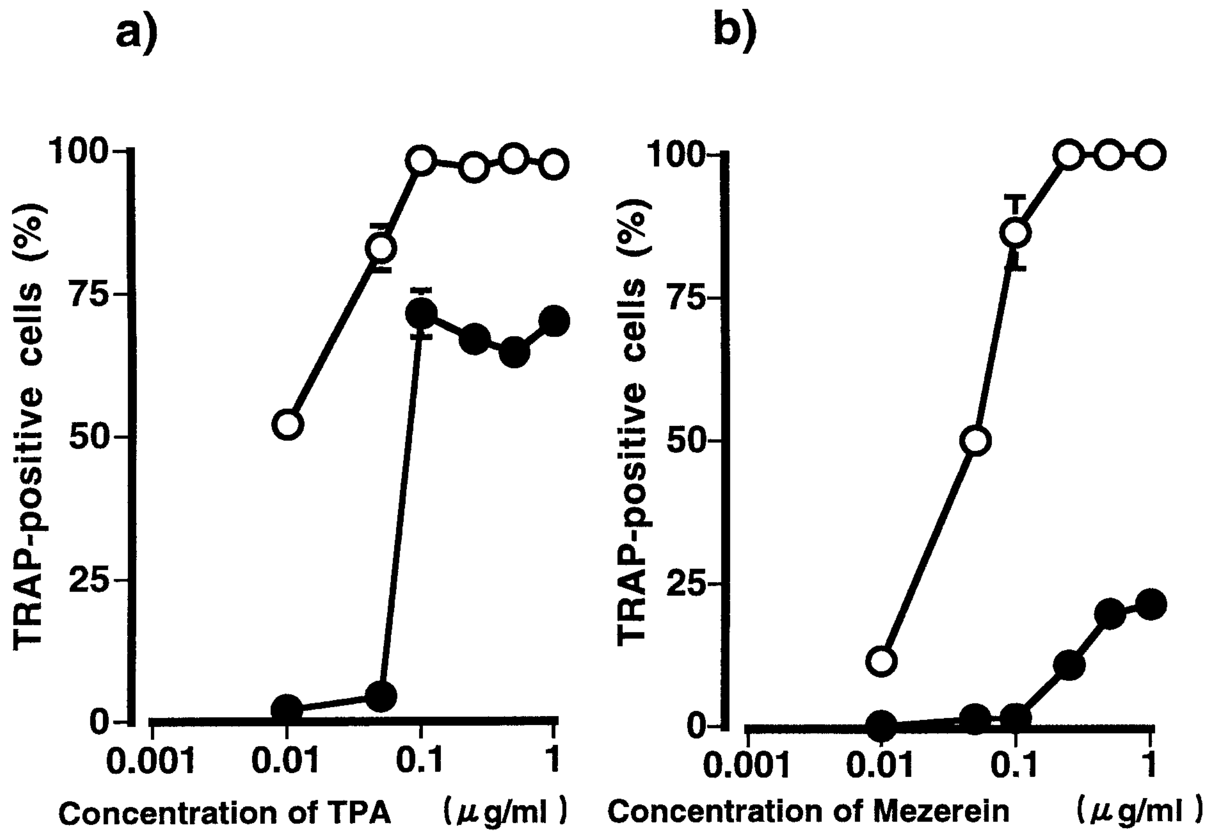
In some experiments, 0.05–1.0 μg/ml mezerein or 4α-phorbol 12,13-didecanoate was used in place of TPA. Human IFN-γ (100–1,000 U/ml) or dexamethasone (10^{-9}–10^{-6} M) was added together with 1,25-$(OH)_2D_3$. H7 (10^{-8}–10^{-5} M) was added 30 min prior to TPA to examine the effect on TRAP-positive cell formation.

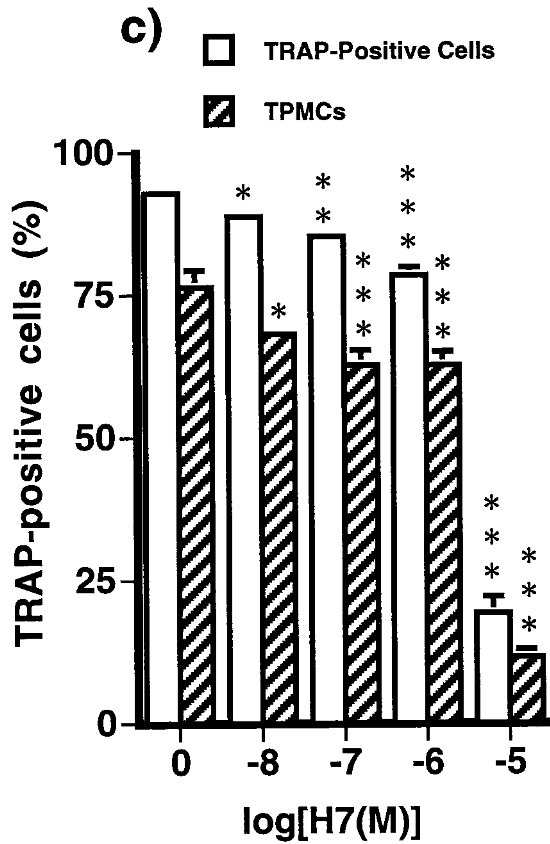
Enzyme Histochemistry

Tartrate-resistant acid phosphatase (TRAP) which was used as a marker for osteoclasts, and nonspecific esterase was used as a marker enzyme for mononuclear phagocytes to determine cell types by means of a previously reported method [Takahashi et al., 1988].

Preparation of ^{45}Ca-Labeled Bone Powder

Labeled bone powder was prepared essentially according to a previously described method [Burger et al., 1984]. Two male mice (C3H/He, 3w) were injected subcutaneously with 100 μCi $^{45}CaCl_2$ every 2 days for a 10-day period. Long bones were removed, cleaned of adhering tissue and bone marrow, chopped into pieces, and washed with PBS two times. These pieces were then digested with proteinase K (10 mg/ml) at 37°C for 4 h, washed twice with PBS, and rinsed twice with 70% ethanol. Bone fragments were dried at 45°C overnight and ground to a coarse powder in a ball mill. Bone powder was suspended in PBS and the fine powder was eliminated by repeated suspension-decantation. The coarse powder remaining was rinsed twice with 70% ethanol and dried under sterile


a)
b)
TRAP-positive cells (%)
100
75
50
25
0
0.001
0.01
0.1
1
Concentration of TPA (μg/ml)
Concentration of Mezerein (μg/ml)


c)
TRAP-Positive Cells
TPMCs
TRAP-positive cells (%)
100
75
50
25
0
0
-8
-7
-6
-5
log[H7(M)]

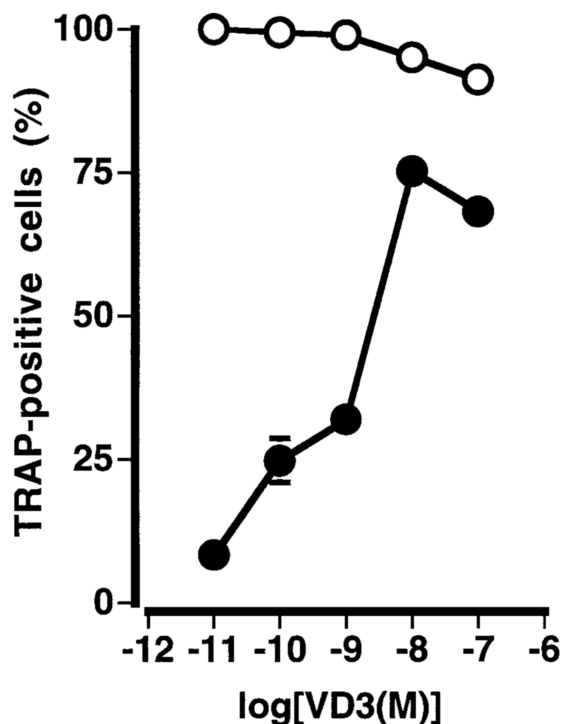


Fig. 4. Effects of 1,25-$(OH)_2D_3$ (VD3) on TPMC formation. ○, TRAP-positive cells; ●, TPMCs. Bars = S.E. for quadruplicate assays.

conditions. The powder was then suspended in PBS at a concentration of 1 mg/ml, and 0.1 ml (1.68×10^5 cpm) of the suspension was added to the culture dish (35 mm) which contained 2.0 ml of medium.

RNA Preparation and the Reverse-Transcribed Polymerase Chain Reaction (RT-PCR)

Total RNA from cultured cells was prepared according to the single-step method of acid guanidinium thiocyanate-phenol-chloroform extraction, and RT-PCR was carried out as previously described [Kurata et al., 1995]. Oligonucleotides corresponding to bases 799–816 and 1,206–1,185 of the human c-fms gene (accession no. x03663), 111–131 and 539–519 of human M-CSF (accession no. M64592), 336–356 and 552–531 of human c-src (accession no. M34469), 675–697 and 1,157–1,140 of human carbonic anhydrase II (accession no. J03037), and 785–806 and 1,035–1,015 of human major histocompatibility complex class I (MHC I) (accession no. M35444) were used as primers.

Fig. 3. Effects of TPA, mezerein, and H7 on TPMC formation. Cells were treated, incubated, and stained as described in the legend for Figure 2 except for the TPA concentration used. **a:** TPA at 0.01 to 1.0 mg/ml was added instead of 0.1 mg/ml. **b:** Mezerein (0.01 to 1.0 mg/ml) was added instead of 0.1 mg/ml TPA. **c:** H7 (10^{-8} to 10^{-5} M) was added to the standard culture medium throughout the incubation period. TRAP-positive cells (○) or TPMCs (●) were counted. Bars = S.E. for quadruplicate assays. $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$ (vs. H7 0 M).

RESULTS

TRAP-Positive Multinuclear Cell Formation

U937 cells treated with TPA (0.1 μg/ml) for 48 h progress to matured macrophages within 3 or 4 days and continue as functional macrophages for more than 2 weeks under normal culture conditions [Hosoya and Marunouchi, 1992], whereas when these cells are incubated in the presence of 1,25-$(OH)_2D_3$ (10^{-8} M) for 5 days after treatment with TPA, TRAP-positive multinucleated cells (TPMCs) appear as shown in Figure 1b. When cells were treated with TPA for 2 days followed by fixing and staining, no TRAP-positive cells appeared (Fig. 1c). The time course of formation of TPMC is shown in Figure 2a. When cells were incubated without adding 1,25-$(OH)_2D_3$, TRAP-positive cells appeared but most contained only a single nucleus (Figs. 1a, 2b). When TPA was added throughout the incubation period, the formation of TPMC was reduced significantly (Fig. 2a). By increasing the concentration of TPA, the efficiency of formation of either TRAP-positive cells or TPMCs increased dose dependently to 0.1 μg/ml and this high level was maintained, as shown in Figure 3a. Almost all of the cells were positively stained for non-specific esterase at 2 days after TPA treatment (data not shown). When 4α-phorbol-12,13-didecanoate, which is a homologue of TPA but not an activator of protein kinase C (PKC), was used instead of TPA, neither macrophages nor TPMC appeared. When mezerein, a nonphorbol ester PKC activator, was added instead of TPA, both TRAP-positive cells and TPMCs appeared and results are shown in Figure 3b. Mezerein induced TRAP-positive cells as effectively as TPA, however, its ability to form TPMC was 1⁄10 to 1⁄5 that of TPA. TRAP-positive cell formation was reduced when H7 was added 30 min prior to TPA addition at a concentration of more than 10^{-8} M and TPMC-formation was inhibited at 10^{-5} M (Fig. 3c). These results suggested that stimulation of PKC was necessary to induce TPMC formation but was not sufficient in itself.

The addition of 1,25-$(OH)_2D_3$ was indispensable for TPMC formation but its addition before

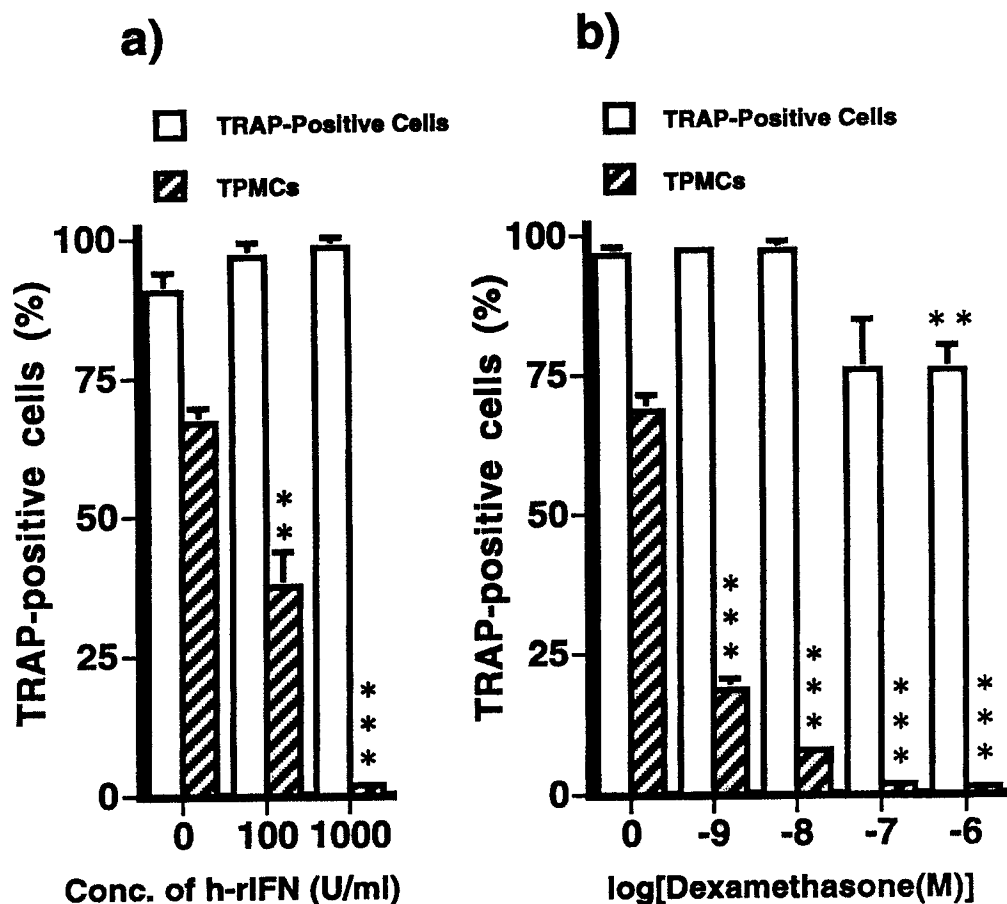


Fig. 5. Inhibitory effects of IFN-γ or dexamethasone on TPMC formation. **a:** IFN-γ (0, 100, 1,000 U/ml) was added to the culture medium. **b:** Dexamethasone (0, 10^{-9} to 10^{-6} M) was added to the culture medium. □, TRAP-positive cells; ▨, TPMCs. Bars = S.E. for quadruplicate assays. $^{***}P < 0.001$; $^{**}P < 0.01$ (vs. no addition of IFN-γ or dexamethasone).

or during TPA treatment had no effect (data not shown). Addition of 1,25-$(OH)_2D_3$ was most effective when it was added after a 48 h incubation of cells with TPA. The efficiency of TPMC formation increased with an increase in the concentration of 1,25-$(OH)_2VD_3$, to 10^{-8} M and then decreased (Fig. 4). Various substances, such as IFN-γ, granulocyte macrophage colony stimulating factor, hepatocyte growth factor, dexamethasone, parathyroid hormone, and insulin, were examined for their effects on TPMC formation. Coculture with ST2 derived from bone marrow stroma cells was also examined [Udagawa et al., 1989]. IFN-γ and dexamethasone significantly inhibited TPMC formation (Fig. 5) but the other substances had no obvious effects. These two substances impaired the effect of 1,25-$(OH)_2D_3$ and restored the expression of M-CSF mRNA, as will be described below.

Characterization of TPMCs

We examined the Ca^{2+} resorptive activity of TPMCs as a functional marker for osteoclasts, since TRAP and possession of a multinucleus are not sufficient for osteoclastic differentiation [Hattersley and Chambers, 1989a]. Results are summarized in Figure 6. ^{45}Ca-labeled particles were added to the standard culture which had been incubated with or without 1,25-$(OH)_2D_3$ for 4 days after TPA treatment. A culture supernatant was obtained after 24 h and assayed for solubilized ^{45}Ca. Cells cultured with 1,25-$(OH)_2D_3$ released 1.5 to 1.7 times more ^{45}Ca than those without 1,25-$(OH)_2D_3$, strongly suggesting that TPMCs have bone resorptive activity. Since the calcitonin receptor is also an important osteoclast marker [Hattersley and Chambers, 1989b], we determined whether calcium releasing activity was sensitive to calcito-

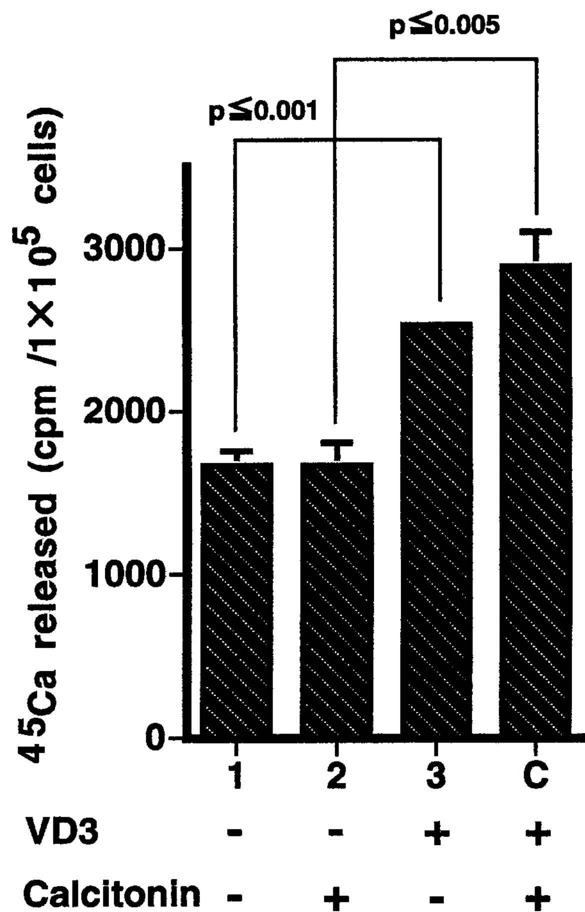


Fig. 6. ^{45}Ca released from ^{45}Ca-bone by TPMC. Cells treated with TPA were cultured with (3 and 4) or without (1 and 2) 1,25-$(OH)_2D_3$ (VD3) for 4 days, incubated with ^{45}Ca-labeled bone particles for 24 h, and the ^{45}Ca released into the culture medium was then measured. Calcitonin (5 ng/ml) was added together with bone particles (2 and 4). n = 6 in each sample. $^{***}P < 0.001$; $^{**}P < 0.005$ (Mann-Whitney U-test).

nin. As shown in Figure 6, the addition of synthetic thyrocalcitonin (5 ng/ml) together with bone powder did not affect ^{45}Ca releasing activity, suggesting that these TPMCs were not calcitonin sensitive.

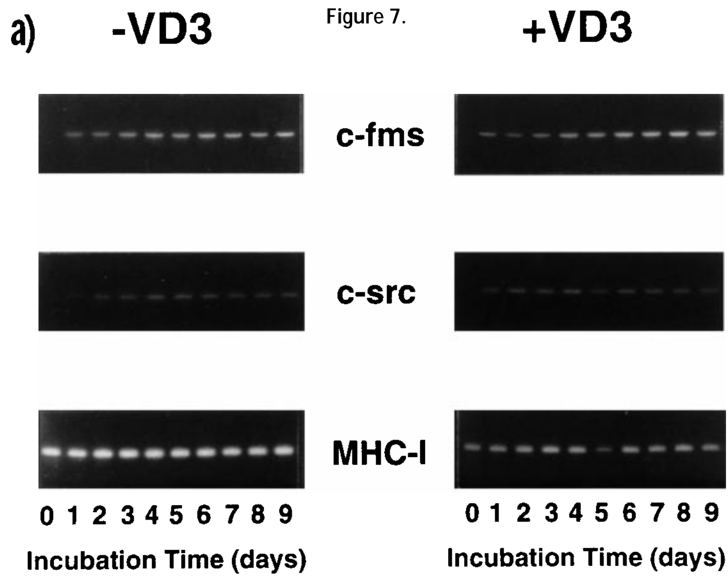
In order to understand more about the nature of TPMCs, we studied the expression of genes related to osteoclast formation. RNA was extracted from U937 cells treated with TPA and cultured with or without 1,25-$(OH)_2D_3$, and RT-PCR was carried out using primers of c-fms, M-CSF, c-src, and carbonic anhydrase II as well as that of MHC-I as a control. We performed each experiment at least three times and found a common tendency in changes in gene expression. Typical results are shown in Figure 7. Transcription of c-fms and c-src was activated by TPA and maintained for 9 days, irrespective of the presence or absence of 1,25-$(OH)_2D_3$, even after the washing out of TPA at 2 days (Fig. 7a). Transcription of CAII was not detected at all (data not shown). The expression of M-CSF was noteworthy in that M-CSF transcription was activated by the addition of TPA, attained a maximum at 4 days, and could be detected for as long as 9 days, although it decreased slightly in the later days of this period (Fig. 7b, lane 1). With the addition of 1,25-$(OH)_2D_3$, however, the expression was attenuated and was detected only at 3 to 5 days (Fig. 7b, lane 2), suggesting some relationship with formation of TPMC. RNA was prepared from cells cultured under various conditions, and the expression of c-fms, c-src, and M-CSF was compared. The expression of c-fms and c-src under various conditions was essentially the same as that shown in Figure 7a, while M-CSF expression changed significantly (Fig. 7b). The rate of formation of TPMC at 9 days is also shown in the same figure.

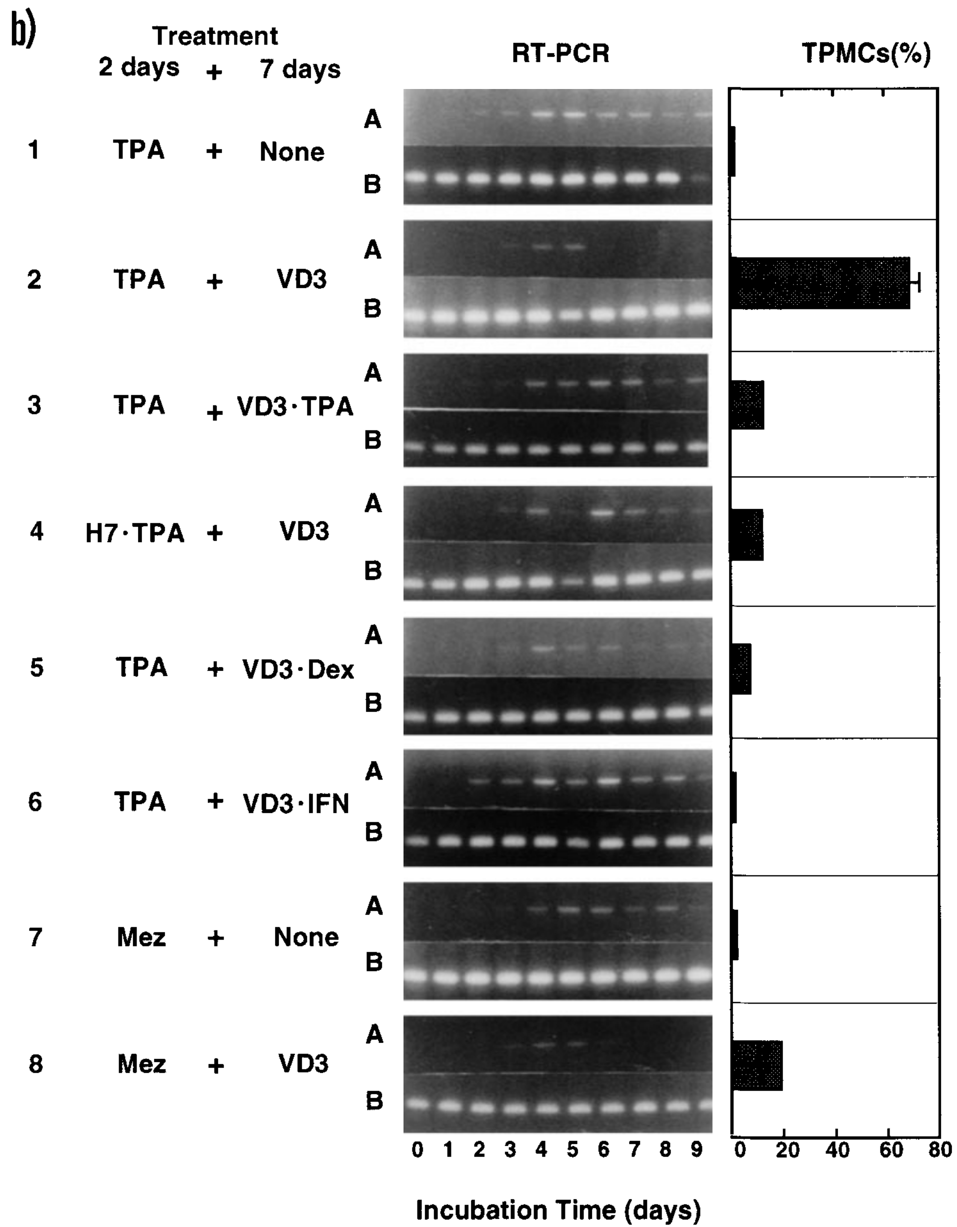
When TPA was added throughout the experiment, M-CSF was expressed for 9 days, as in the case when cells were incubated without adding 1,25-$(OH)_2D_3$ (Fig. 7b, lane 3). With the addition of dexamethasone or IFN-γ during 1,25-$(OH)_2D_3$ treatment, the expression of M-CSF recovered and was detectable for 9 days, although in the case of dexamethasone, expression of M-CSF was slightly less than with IFN-γ (Fig. 7b, lanes 5 and 6). When mezerein was used instead of TPA, M-CSF was detected from 3 to 9 days as was seen in the TPA treatment (Fig. 7b, lane 7). Furthermore, when 1,25-$(OH)_2D_3$ was added afterwards, M-CSF expression was attenuated again but detected from 3 to 6 days, a slightly longer period than that observed in the case of TPA (Fig. 7b, lane 8). Cells treated with 1,25-$(OH)_2D_3$, dexamethasone, and IFN-γ alone proliferated without differentiation. By the treatment with H7 (10^{-5} M) prior to TPA, formation of TPMC was inhibited (Fig. 3c) but M-CSF expression was detected (Fig. 7b, lane 4). These results suggested a strong correlation between the attenuation of M-CSF transcription and the formation of TPMC.

DISCUSSION

Successive treatment with TPA and 1,25-$(OH)_2D_3$ resulted in the formation of TRAP-positive multinucleated cells (TPMCs) from U937. These TPMCs exhibited bone resorptive

Figure 7.





activity, although they did not respond to calcitonin. Because of this activity, we referred to these TPMCs as osteoclast-like cells. Under various experimental conditions, we examined the expression of genes related to osteoclast formation. Treatment with TPA resulted in the activation of c-fms, c-src, and M-CSF, however, treatment with 1,25-$(OH)_2D_3$ was found to suppress M-CSF expression using our standard procedures. This repressive effect by 1,25-$(OH)_2D_3$ was blocked by dexamethasone, IFN-γ, and prolonged exposure to TPA. These three treatments also inhibited TPMC formation, suggesting a strong correlation between this formation and transient expression of M-CSF. Considering also that the *op/op* mouse, which harbors a defective M-CSF gene, can produce neither macrophages nor osteoclasts [Felix et al., 1990; Yoshida et al., 1990], M-CSF appears indispensable for the normal development of these types of cells. Our results show that M-CSF is only necessary for TPMC formation during a limited phase of differentiation. It was also reported that in another leukemic cell line, HL60, TPA-activated expression of c-*fms* required attenuation by 1,25-$(OH)_2D_3$ for the formation of osteoclast-like cells [Biskobing and Rubin, 1993]. Therefore, in the normal development of the macrophage/osteoclast type of cell, effective expression of M-CSF would be temporarily and spatially regulated.

Kukita et al. [1992] reported that TRAP-positive, bone resorptive multinucleated cells were formed in rat bone marrow cells cultured in the presence of 10^{-9} M TPA. However, in the presence of 10^{-7} M TPA, TRAP-negative multinucleated cells without bone resorbing activity were formed. Hattersley and Chambers [1989a] reported that peritoneal macrophages became TRAP positive and that multinucleated cells were incapable of bone resorption, whereas cells from bone marrow became bone resorptive TRAP-positive under the same conditions. These results suggest that the expression of various osteoclast phenotypes is regulated independently and that the expression of calcitonin receptor which was defective in the TPMCs formed in the present study could be induced by treatment with other factors at a suitable stage of differentiation. In this context, although coculture of U937 cells with ST2 did not activate the formation of TPMCs, other kinds of stromal cells, such as stromal-endothelial cells derived from human bone marrow, would activate other genes characteristic of osteoclasts, as in the case of M1 and mouse stromal cells [Benayahu et al., 1994].

Fig. 7. Expression of c-fms, c-src, and M-CSF in TPMCs. **a:** Cells were cultured according to the standard procedure with or without 1,25-$(OH)_2D_3$ (VD3) for 9 days and RT-PCR was carried out using RNA prepared as described in Materials and Methods. Results obtained using MHC-I primer as a control are shown. **b:** Cells were cultured according to the indicated conditions and RT-PCR was carried out. Results regarding M-CSF expression are shown (A of each lane), together with loaded controls (B of each lane). In the panel on the right, the rate of TPMC formation (%) under each experimental condition is summarized. VD3 · TPA, VD3 · Dex, and VD3 · IFN indicated that cells were treated with TPA (0.1 μg/ml), dexamethasone (10^{-8} M), and IFN-γ (1,000 U/ml) together with VD3 (10^{-8} M), respectively. H7 · TPA, H7 (10^{-5} M) was added 30 min prior to TPA addition. Mez, cells were treated with mezerein (0.5 μg/ml) instead of TPA for 2 days.

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