Effects of 1α,25-Dihydroxyvitamin D3 on Macrophage Colony-Stimulating Factor Production and Proliferation of Human Monocytic Cells

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1α,25-Dihydroxyvitamin D3 [1α,25(OH)2D3] stimulates the proliferation of human monocytes in vitro. In the present study, we investigated a possible role of macrophage colony-stimulating factor (M-CSF) in 1α,25(OH)2D3-induced proliferation of human circulating monocytes and the effects of 1α,25(OH)2D3 on M-CSF production by human monocytic cells. Both 1α,25(OH)2D3 and recombinant human M-CSF increased 2.5-fold the number of human circulating monocytes on day 6 of the culture. These effects were inhibited by antihuman M-CSF antibody as well as by anti-αfms antibody, although these antibodies themselves did not affect the number when added to control culture. These results indicated that M-CSF is required for 1α,25(OH)2D3-stimulated monocyte proliferation. In addition, 1α,25(OH)2D3 stimulated M-CSF secretion from human circulating monocytes. Secretion and mRNA expression of M-CSF by 12-O-tetradecanoylphorbol-13-acetate (TPA)-treated THP-1 cells (human monomyelocytic leukemia cell line) and TPA-treated HL-60 cells (human promyelocytic leukemia cell line) were also increased by 1α,25(OH)2D3. M-CSF secretion from TPA-treated THP-1 cells was increased by 1α,25(OH)2D3 in a dose-dependent and metabolite-specific manner. The present study demonstrates that 1α,25(OH)2D3 is a potent stimulator for M-CSF production by human monocytic cells and that the proliferative effect of 1α,25(OH)2D3 on human monocytes may be attributed, at least in part, to the stimulated secretion of M-CSF.

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MATERIALS AND METHODS

Reagents and cDNA probe. 1α,25(OH)2D3 was kindly provided by Feijin Limited (Tokyo, Japan). 1α(OH)D3 was a kind gift from Dr Yasushi Okamoto of Nisshin Flour Milling Co Ltd (Tokyo, Japan). 24R,25(OH)2D3 was kindly provided by Kureha Chemical Industry Co Ltd (Tokyo, Japan). Cholecalciferol (vitamin D3), 12-O-tetradecanoylphorbol-13-acetate (TPA), Triton-X, and naphthol blue black were purchased from Sigma Chemical (St Louis, MO). 25(OH)D3 was purchased from Wako Pure Chemical Industries (Osaka, Japan). 1α,25(OH)2D3 and other derivatives of vitamin D3 were dissolved in ethanol and the final concentration of ethanol in culture medium was 0.01% or less. The culture medium containing 0.01% of ethanol had no effect on either M-CSF secretion or proliferation of cells. Recombinant human M-CSF (rhM-CSF) was kindly provided by Dr Eiji Takahashi of Morinaga Milk Industry Co Ltd (Tokyo, Japan). rhM-CSF and cDNA probe for human M-CSF were prepared as described previously. Colony-stimulating activity of rhM-CSF was determined by a monolayer agar culture system containing mouse unfractionated bone marrow cells, with 1 unit defined as the amount needed to form a colony. The specific activity of rhM-CSF was equivalent to 106 U/mg. Recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) with 5 × 105 U/...
mg, recombinant human granulocyte colony-stimulating factor (rhG-CSF) with 10³ U/mg, and recombinant human interleukin-3 (rhIL-3) with 2.5 × 10⁴ U/mg were purchased from Genzyme (Boston, MA).

Antibodies. Antihuman M-CSF (anti-hM-CSF) antibody, which is rabbit polyclonal IgG, was kindly provided by Dr Munec Yamada of Morinaga Milk Industry Co, Ltd. Anti-hM-CSF was prepared as previously reported.21 The neutralizing activity of anti-hM-CSF antibody was 3 × 10³ U/mg, which was determined by mouse bone marrow colony formation assay as described below. Monoclonal mouse antihuman GM-CSF antibody (anti-hGM-CSF antibody; Genzyme)21 and monoclonal mouse antihuman IL-1β antibody (anti-hIL-1β antibody; Genzyme)21 were reported to demonstrate activities to neutralize each factor. Anti-c-fms/M-CSF receptor antibody (anti-c-fms antibody; Oncogene Science, Uniondale, NY) was reported to inhibit human M-CSF activity.28

Cell culture. Human monocytes were prepared as described by Hosoi et al.,26 with minor modifications. Briefly, mononuclear cells were prepared from freshly drawn heparinized venous blood of healthy adult nonsmoking volunteers by Ficoll-Paque (Pharmacia, Uppsala, Sweden). After the mononuclear cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ - 95% air for 1 hour, nonadherent cells were removed by rinsing three times with phosphate-buffered saline (PBS). Adherent cells were then detached by pipetting. The cells were resuspended in the complete medium; RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 10% (vol/vol) of heat-inactivated fetal bovine serum (FBS; Bioserum, Victoria, Australia), 15 mmol/L HEPES (pH 7.4), and 80 mg/L of kanamycin monosulfate. Two-hundred-microliter aliquots of cell suspension (10⁶ cells/mL) were seeded onto 96-well tissue culture plates (Falcon, Lincoln Park, NJ). More than 95% of harvested cells were identified to be monocytes by Wright-Giemsa staining and nonspecific esterase staining. Nonspecific esterase staining was performed using a commercial kit (Muto Pure Chemical, Tokyo, Japan).

TPH-1 and HL-60 cells, provided by Japanese Cancer Research Resources Bank (Tokyo, Japan), were cultured in RPMI 1640 supplemented with 15 mmol/L HEPES, 80 mg/L of kanamycin monosulfate, and 5% or 10% FBS, respectively. TPH-1 and HL-60 cells (10⁶ cells each) in 2 mL of the medium were inoculated onto 6-well tissue culture plates (Falcon) and cultured in the presence or absence of TPA and/or 1α,25(OH)₂D₃ at the concentration indicated in the figure legends. We used TPA-treated TPH-1 cells and TPA-treated HL-60 cells as an experimental model to investigate the details of the effects of 1α,25(OH)₂D₃ on M-CSF production by monocyte/macrophages. TPA is known to induce growth arrest and the phenotypic changes in THP-127 and HL-60 cells24 into those of differentiated macrophages such as capacity of phagocytosis, adherence to the stratum, and c-fms expression.

Assay of M-CSF. The concentration of M-CSF in the culture supernatants was measured by an enzyme-linked immunosorbent assay (ELISA) specific for human M-CSF. The details of the assay system were previously reported.21 The minimal detectable level of M-CSF in our assay system was 0.1 ng/mL.

Colony formation assay. The neutralizing activity of anti-hM-CSF antibody was determined according to the method described by Motoyoshi et al.21 with minor modifications. Briefly, serially diluted antibody was mixed with 100 U of rhM-CSF and incubated for 1 hour at 37°C before performing the mouse bone marrow colony formation assay. The neutralizing units were calculated from the dilution fold of antibody, which resulted in the formation of half the number of colonies in the control experiment.

To ascertain the specificity of anti-hM-CSF antibody, effects of anti-hM-CSF antibody on rhGM-CSF-, rhG-CSF-, and rhIL-3-dependent colony formations were examined according to the method described by Motoyoshi et al.21 Briefly, human bone marrow cells were obtained by sternal puncture from healthy volunteers who gave informed consent. After incubating each factor with anti-hM-CSF antibody or nonimmunized rabbit IgG for 1 hour at 37°C, rhGM-CSF or rhG-CSF or rhIL-3 was added to the monolayer culture system containing human monocyte-depleted bone marrow cells. The number of colonies formed during the 7-day culture was determined.

Determination of nucleus number of human monocytes. The nucleus number of human monocytes was counted according to the method described by Nakagawara et al.,29 with minor modifications. Monocytes were cultured in 96-well tissue culture plates for 6 days, and then the cells were washed four times with 200 µL of 0.95% (wt/vol) saline. To stain the nucleus, 50 µL of 1% (wt/vol) Triton X-100 in 0.1 mol/L citric acid with 0.05% (wt/vol) naphthol blue black was added.

RNA isolation and Northern blot analysis. Total RNA of TPH-1 and HL-60 cells was obtained by acid guanidinium thiocyanate-phenol-chloroform extraction.30 Twenty micrograms of RNA from TPH-1 cells or 10 µg from HL-60 cells was electrophoretically separated on a 1% agarose gel containing 2.2 mol/L formaldehyde in MOPS buffer (20 mmol/L, 2-[N-morpholino]propanesulfonic acid, pH 7.0, 5 mmol/L sodium acetate, and 0.5 mmol/L EDTA) under denaturing condition, and was blotted to a nylon filter (GeneScreen Plus; NEN Research Products, Boston, MA). The 3²P-labeled human M-CSF cDNA probe, prepared by random oligonucleotide primer method, was denatured and then added to the hybridization fluid. Hybridization was performed overnight at 65°C. The filters were washed for 5 minutes at room temperature in 50 mmol/L phosphate buffer (pH 7.4), followed by a wash for 20 minutes at 65°C. The filters were exposed to x-ray film (X-OMAT; Eastman Kodak, Rochester, NY) with intensifying screens for 3 days at -80°C. To normalize RNA concentration, the filters were dehydrized in boiling 0.1% sodium dodecyl sulfate (SDS) in 50 mmol/L phosphate buffer (pH 7.4) and then rehybridized with radiolabeled mouse β-actin cDNA probe. The filters were again exposed to x-ray films with intensifying screens for 1 day at -80°C.

Quantification of specific RNA. Autoradiograms were scanned by EPSON GT-6000 (Seiko-Epson, Tokyo, Japan). The intensity of signals was quantitated with NIH Image program version 1.44 using an Apple Macintosh computer (Cupertino, CA). Levels of specific mRNA were normalized by the level of hybridizing β-actin mRNA and were expressed as a percentage of control.

Statistical analysis. Statistical significance between two groups was analyzed by unpaired Student’s t-test. Statistical analysis of dose dependency of the effects of vitamin D derivatives and anti-c-fms antibody was performed by using one-factor analysis of variance, with Duncan’s test for multiple comparisons. A P value of less than 0.05 was considered significant. All data were presented as mean ± SEM.

RESULTS

Effects of 1α,25(OH)₂D₃ on proliferation of cultured monocytes. As shown in Fig 1, 1α,25(OH)₂D₃ increased in the nucleus number of monocytes in a dose-dependent manner. A statistically significant increase was observed at 10⁻¹⁰ mol/L and higher concentrations and maximal increase was obtained at 10⁻⁸ mol/L. Multinucleated cells were scarcely observed during the 6-day culture.

Effects of anti-hM-CSF antibody on 1α,25(OH)₂D₃-induced increase in the nucleus number of human monocytes. To elucidate a possible role(s) of cytokines, which are known to induce proliferation or prolongation of survival of human monocytes, the effects of anti-hM-CSF antibody, anti-hGM-
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Fig 1. Effects of 1α,25(OH)2D3 on nucleus number of monocytes. Human peripheral blood mononuclear cells were obtained by Ficoll-Paque gradient from heparinized blood as described in Materials and Methods. Monocytes were isolated from mononuclear cells by adhesion and seeded onto 96-well tissue culture plates (2 × 104 cells/dish). On days 2 and 4, the medium was changed into a fresh one with or without the indicated concentration of 1α,25(OH)2D3. On day 6, the numbers of nuclei of adherent cells were counted as described in Materials and Methods. Bar graphs indicate the mean of the results of three independent experiments (±SEM) that were performed in triplicate cultures using human monocytes from different donors.

Table 1. Effects of Anti-hM-CSF Antibody in 1α,25(OH)2D3-Induced Increases in Nucleus Number

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of Control</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>1α,25(OH)2D3 (10⁻⁴ mol/L)</td>
<td>258 ± 18*</td>
</tr>
<tr>
<td>+ anti-hM-CSF antibody (3.3 μg/mL)</td>
<td>137 ± 21†</td>
</tr>
<tr>
<td>1α,25(OH)2D3 (10⁻⁴ mol/L) + anti-hGM-CSF antibody (10 μg/mL)</td>
<td>243 ± 21*</td>
</tr>
<tr>
<td>+ anti-hIL-1β antibody (10 μg/mL)</td>
<td>248 ± 24*</td>
</tr>
<tr>
<td>1α,25(OH)2D3 (10⁻⁴ mol/L) + rabbit IgG (3.3 μg/mL)</td>
<td>260 ± 26*</td>
</tr>
</tbody>
</table>

Human peripheral blood monocytes were seeded onto 96-well tissue culture plates (2 × 10⁴ cells/well). On days 2 and 4, the medium was changed into a fresh one with or without 1α,25(OH)2D3 (10⁻⁴ mol/L). Anti-hM-CSF antibody (3.3 μg/mL), anti-hGM-CSF antibody (10 μg/mL), anti-hIL-1β antibody (10 μg/mL), or rabbit IgG (3.3 μg/mL) was added every day. On day 6, the numbers of nuclei of adherent cells were counted as described in Materials and Methods. Results are expressed as the mean of three independent experiments (±SEM) that were performed in triplicate cultures using human monocytes from different donors.

* P < .01 vs control.
† P < .05 vs 1α,25(OH)2D3 (10⁻⁴ mol/L).

Table 2. Effects of Anti-hM-CSF Antibody on CSF- or Cytokine-Dependent Colony Formation

<table>
<thead>
<tr>
<th>Antibody</th>
<th>rhM-CSF</th>
<th>rhGM-CSF</th>
<th>rhG-CSF</th>
<th>rhIL-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>200 μg/dish</td>
<td>0</td>
<td>98 ± 12</td>
<td>106 ± 17</td>
<td>96 ± 6</td>
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<td>20 μg/dish</td>
<td>0</td>
<td>116 ± 7</td>
<td>108 ± 4</td>
<td>91 ± 7</td>
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<tr>
<td>2 μg/dish</td>
<td>13 ± 10</td>
<td>103 ± 4</td>
<td>98 ± 13</td>
<td>99 ± 4</td>
</tr>
</tbody>
</table>

rhM-CSF, rhGM-CSF, and rhIL-3 were mixed with serially diluted anti-hM-CSF antibody and incubated for 1 hour at 37°C before performing human monocyte-depleted bone marrow culture in 1 mL of semisolid agarose medium. The numbers of colonies formed during the 7-day culture were counted in triplicate cultures. Results are expressed as the mean percentage of control (±SEM), in which each CSF or cytokine was incubated with nonimmunized rabbit IgG (200 μg/dish).
monocytes (1.57 ± 0.08 × 10⁴ nuclei/well) in control culture after 6 days decreased slightly compared with that after 24 hours of incubation.

Effects of anti-c-fms antibody on 1α,25(OH)₂D₃-induced increase in the nucleus number of human monocytes. To examine further whether M-CSF plays a significant role in the stimulatory effect of 1α,25(OH)₂D₃ on monocyte proliferation, the effects of anti-c-fms antibody on 1α,25(OH)₂D₃-induced increase in the nucleus number was studied. As shown in Fig 2, anti-c-fms antibody inhibited both of rhM-CSF (10 ng/mL) and 1α,25(OH)₂D₃ (10⁻⁸ mol/L) induced increase in the nucleus number of monocytes in a dose-dependent manner. On the other hand, anti-c-fms antibody (3.0 μg/mL) did not affect the nucleus number when added to control culture.

Effects of 1α,25(OH)₂D₃ on M-CSF secretion from human monocytes. We studied the effects of 1α,25(OH)₂D₃ on M-CSF secretion, because the results described above indicated that M-CSF is required for 1α,25(OH)₂D₃-stimulated monocyte proliferation. As shown in Fig 3, M-CSF concentration in the supernatants of 1α,25(OH)₂D₃ (10⁻⁴ mol/L)-treated human monocytes was three times higher than that of control cells. On the other hand, no difference was found in nucleus number between 1α,25(OH)₂D₃-treated cells (1.89 ± 0.05 × 10⁴ nuclei/well) and untreated cells (1.92 ± 0.07 × 10⁴ nuclei/well) at 48 hours after the inoculation of the cells.

Effects of 1α,25(OH)₂D₃ on M-CSF secretion from TPA-treated THP-1 cells and TPA-treated HL-60 cells. The effects of 1α,25(OH)₂D₃ and TPA on M-CSF secretion from THP-1 and HL-60 cells were next examined. When TPA was not added to the culture medium, M-CSF was undetectable in the supernatants of either THP-1 cells or HL-60 cells, regardless of whether the cells were cultured with 1α,25(OH)₂D₃ (10⁻⁸ mol/L). On the other hand, M-CSF was detectable when THP-1 cells and HL-60 cells were treated with TPA (100 ng/mL). Simultaneous addition of 1α,25(OH)₂D₃ (10⁻⁸ mol/L) and TPA (100 ng/mL) increased M-CSF secretion from THP-1 cells threefold (Fig 4A) and from HL-60 cells twofold (Fig 4B) compared with the cells treated with TPA alone, respectively.

Once THP-1 cells were treated with TPA (100 ng/mL) for 24 hours, both M-CSF secretion and its responsiveness to 1α,25(OH)₂D₃ continued to be observed even after the
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Fig 4. Effects of 1α,25(OH)2D3 on M-CSF secretion from TPA-treated THP-1 cells (A) and TPA-treated HL-60 cells (B). THP-1 cells and HL-60 cells were seeded at a concentration of 5 × 10^5 cells/mL in 6-well tissue culture plates and cultured with or without 1α,25(OH)2D3 (10^-8 mol/L) in the presence of TPA (100 ng/mL). M-CSF concentrations in the supernatants after 24 or 48 hours of culture were assayed by ELISA. Results are expressed as mean ± SEM for quadruplicate cultures. *P < .05; ***P < .001.

Effects of various concentrations of vitamin D3 derivatives on M-CSF secretion. Figure 6 shows the effects of various concentrations of vitamin D3 derivatives on M-CSF secretion from TPA-treated THP-1 cells. 1α,25(OH)2D3 increased M-CSF secretion in a dose-dependent manner and the plateau level was obtained at 10^-8 mol/L. On the contrary, cholecalciferol was found to be ineffective on M-CSF secretion at any concentrations examined. 1α-hydroxyvitamin D3, 25-hydroxyvitamin D3, and 24R,25-dihydroxyvitamin D3 were much less effective than 1α,25(OH)2D3.

Northern blot analysis of M-CSF. In the absence of TPA, the expression of M-CSF mRNA was not detected in THP-1 cells either treated or not treated with 1α,25(OH)2D3 (10^-8 mol/L) (Fig 7A, lanes 1 and 2). When treated with TPA (100 ng/mL), the expression of M-CSF mRNA was observed at the level of 4 kb (Fig 7A, lane 3; Fig 7B, lane 1). The level of expression was increased by simultaneous addition of TPA and 1α,25(OH)2D3 (10^-8 mol/L) in both THP-1 cells and HL-60 cells (Fig 7A, lane 4; Fig 7B, lane 2). Densitometric analysis showed that the amount of mRNA of M-CSF in 1α,25(OH)2D3-treated THP-1 and HL-60 cells was 3.9 times and 3.1 times greater than that in cells without 1α,25(OH)2D3 treatment, respectively.

DISCUSSION

In the present study, we clearly demonstrated that M-CSF plays a significant role when 1α,25(OH)2D3 exerts its effects on survival and/or proliferation of human monocytes and that 1α,25(OH)2D3 increased M-CSF production by human monocytic cells in vitro.

Both 1α,25(OH)2D3 and rhM-CSF increased the nucleus number of monocytes during the 6-day culture in a dose-dependent manner (Fig 1), consistent with the previous observations.315 1α,25(OH)2D3- and rhM-CSF-induced increases in nucleus number are considered to be attributable not only to prolongation of survival but also to promotion of monocyte proliferation. In the present study, both anti-M-CSF (3.3 µg/mL) antibody (Table 1) and anti-c-fms antibody (3.0 µg/mL) antibody (Fig 2) abolished rhM-CSF–induced increase and inhibited 1α,25(OH)2D3–induced increase in nucleus number. On the other hand, neither of the same concentration of anti-hM-CSF antibody nor anti-c-fms antibody affected the basal level of the nucleus number in control culture. In addition, anti-gM-CSF antibody (10 µg/mL) and anti-IL-1β antibody (10 µg/mL) did not affect 1α,25(OH)2D3–induced increase in nucleus number in the present study (Table 1), although GM-CSF15 was reported to induce the proliferation and IL-1β33 was reported to prevent apoptosis of human circulating monocytes. Taking together these results with the stimulatory effects of 1α,25(OH)2D3,
on M-CSF secretion from human monocytes, M-CSF is considered to play a critical role as an autocrine or paracrine factor in the effect of 1α,25(OH)2D3 on survival and/or proliferation of monocytes in vitro. However, we cannot exclude the possibility that other cytokines and growth factors may be important costimuli, because some cytokines, such as IL-1,42 and monocytes, 54-67 Recent studies demonstrated that M-CSF production by human monocytes is promoted by phorbol ester, 43 cytokines, 43,46 and monoclonal antibodies against surface adhesion molecules. 47 Although Kimura et al 48 reported that progestin stimulates M-CSF synthesis in endometrial adenocarcinoma cells, little is known about the hormonal regulation of M-CSF production.

Therefore, we considered it worthwhile to examine whether 1α,25(OH)2D3 modulates M-CSF synthesis and we studied the effects of 1α,25(OH)2D3 on M-CSF secretion from human monocytes. 1α,25(OH)2D3 increased M-CSF secretion from human peripheral blood monocytes (Fig 1). The active form of vitamin D3 was found to enhance M-CSF secretion from TPA-treated human peripheral blood monocytes (Fig 3). A stimulatory effect of 1α,25(OH)2D3 on M-CSF secretion was also observed in TPA-treated THP-1 cells and TPA-treated HL-60 cells (Fig 4). The mRNA expression of M-CSF in TPA-treated leukemia cell lines was augmented by 1α,25(OH)2D3, as well (Fig 7). The active form of vitamin D3 was found to enhance M-CSF secretion from TPA-treated THP-1 cells in a dose-dependent and metabolite-specific manner (Fig 6). Among the vitamin D3 derivatives tested, 1α,25(OH)2D3 was the most effective, followed successively by 1α(OH)D3, 25(OH)D3, and 24R,25(OH)2D3. Cholecalciferol (vitamin D3) had no effect. The potency of various vitamin D3 derivatives in promoting M-CSF secretion ap-

Fig 5. The lasting effect of TPA treatment on M-CSF secretion and its responsiveness to 1α,25(OH)2D3. THP-1 cells (5 × 10⁵ cells/mL) were treated with TPA (100 ng/mL) for 24 hours (the first 24-hour incubation, a and b). We then washed the cells three times with PBS and changed the medium into a fresh one without TPA. We continued to incubate the cells until 24 hours (the second incubation, c and d), 48 hours (the third incubation, e and f), and 72 hours (the fourth incubation, g and h) after the end of TPA treatment. During the incubation, washing and medium change were performed in the same way every 24 hours. Supernatants were collected from the first 24-hour incubation (a and b), the second 24-hour incubation after the first medium change (c and d), the third 24-hour incubation after the second medium change (e and f), and the fourth 24-hour incubation after the third medium change (g and h). In the culture of (b), (d), (f), and (h), 1α,25(OH)2D3 (10⁻⁸ mol/L) was added to the medium only during the last 24-hour incubation of each culture. On the other hand, the cells were never treated with 1α,25(OH)2D3 in the culture of (a), (c), (e), and (g). M-CSF concentrations in the supernatants were determined by ELISA. Results are expressed as mean ± SEM for quadruplicate cultures. ***P < .001; N.D., not detected (<0.1 ng/mL).

Fig 6. Effects of various concentrations of vitamin D3 derivatives on M-CSF secretion from TPA (100 ng/mL) treated THP-1 cells. THP-1 cells were seeded at a concentration of 5 × 10⁵ cells/mL and cultured with designated concentration of vitamin D3 derivatives in the presence of TPA (100 ng/mL). The supernatants were collected after 48 hours of incubation and M-CSF concentrations were measured by ELISA. Results are expressed as mean ± SEM for quadruplicate cultures.
pears to be parallel with their binding affinity to the specific cytosol receptors (VDR) found in the intestine and in HL-60 cells. These results are consistent with the notion that 1α,25(OH)₂D₃ exerts its effects on TPA-treated THP-1 cells through specific receptor (VDR)-mediated events, similar to those observed in classic vitamin D target tissues. However, it remains unclear whether 1α,25(OH)₂D₃ induces enhanced transcription of M-CSF mRNA directly or not, because no typical vitamin D-responsive element (VDRE) sequences are found within 400 nucleotides upstream of the M-CSF gene. The possibilities that 1α,25(OH)₂D₃ induces other factors that enhance M-CSF synthesis or that 1α,25(OH)₂D₃ stabilizes mRNA of M-CSF should be taken into account.

We observed that THP-1 and HL-60 cells, but not monocytes, required prior or concurrent treatment with TPA for production of M-CSF and enhanced M-CSF expression in response to 1α,25(OH)₂D₃. Concerning M-CSF secretion and its responsiveness to 1α,25(OH)₂D₃, the effect of TPA treatment continued until the 24-hour incubation after the second medium change into that without TPA (Fig 5e and f). However, M-CSF was not detected after the third medium change either in the cells treated with or without 1α,25(OH)₂D₃ (10⁻⁸ mol/L) (Fig 5g and h). During the 24-hour incubation after the third medium change, most of the cells detached from the plates and started to proliferate again (Kaneki, October 1993, unpublished observation). Hass et al. reported the "retrodifferentiation" of TPA-pretreated U937 cells (human monoblastic leukemia cell line) from static, adherent cells with the monocytic phenotype into cycling, nonadherent cells with undifferentiated phenotype after several replacements with TPA-free medium. It is possible that M-CSF secretion and its responsiveness to 1α,25(OH)₂D₃ reflects the cellular state of differentiation of THP-1 cells. On the other hand, our preliminary experiment using ³H-labeled TPA showed that 19% of TPA added primarily to the culture remained associated with the cells after the first wash and 5.5% remained after the second wash. Therefore, we speculate that the continuous presence of a substantial amount of TPA might be required for THP-1 cells not only to synthesize M-CSF synthesis and respond to 1α,25(OH)₂D₃ but also to stay in the cellular state with the phenotype of differentiated macrophages.

In conclusion, we found that proliferative effects of 1α,25(OH)₂D₃ on human cultured monocytes were inhibited by anti-hM-CSF antibody or anti-c-fms antibody and that 1α,25(OH)₂D₃ stimulates M-CSF synthesis by human monocytic cells. These results suggest that 1α,25(OH)₂D₃ exerts its effect on monocyte/macrophages, at least in part, through
M-CSF in an autocrine or paracrine manner and exerts some of its diverse effects by modulating the monokine network. However, further studies are required to clarify the biologic significance of the stimulatory effect of 1α,25(OH)2D3 on M-CSF production in physiologic conditions and also in particular conditions such as pregnancy or disorders with granulomatous histology.

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