Synergistic Effect of Granulocyte-Macrophage Colony-Stimulating Factor and 1,25-Dihydroxyvitamin D3 on the Differentiation of the Human Monocytic Cell Line U937

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The human monoblastlike cell line U937 can be induced to differentiate by a variety of agents including γ-interferon, phorbol esters, retinoic acid, and 1,25-dihydroxyvitamin D3 (VD3). Incubation of U937 with 1 to 1,000 units of recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) did not induce macrophage differentiation. A synergistic effect on macrophage differentiation was observed, however, when U937 was cocultured with 10⁻⁶ mol/L VD3 plus 50 U/mL GM-CSF. GM-CSF plus VD3-treated cells demonstrated significant increases in OKM1 antigen expression, increased chemokinesis and chemotaxis, and increased Fc receptor-mediated erythrophagocytosis. Human peripheral blood monocyte cultures also demonstrated increased OKM1 antigen expression and chemotaxis when incubated with 50 to 500 U/mL of GM-CSF for 48 to 72 hours. VD3, however, was not necessary for the increases in effector function observed for GM-CSF-stimulated monocyte cultures. In distinction to the synergistic effect of GM-CSF on VD3-induced differentiation of U937, recombinant human granulocyte colony-stimulating factor (G-CSF) at comparable concentrations had no augmenting effect over that observed for VD3 alone. These results suggest that GM-CSF, in the presence of other physiological stimuli, can induce significant phenotypic changes in GM-CSF-nonresponsive cells of the monocytic lineage and can increase the effector functions of GM-CSF-responsive peripheral blood monocyte cultures.

**SYNOPSIS**

**MATERIALS AND METHODS**

**Cells.** The human histiocytic cell line U937 (provided by Dr Stephen Krane, Department of Medicine, Harvard Medical School, Boston) was maintained as a continuous culture in RPMI 1640 with 10% fetal calf serum. Human peripheral blood monocytes were prepared by Ficoll-Hypaque gradient separation of mononuclear cells followed by the removal of nonadherent cells. Monocytes were maintained in culture for three to five days in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. U937 at 2 x 10⁵ melanoma cell line. GM-CSF receptors have been partially characterized on human and murine cell populations. The human receptor has been detected on neutrophils, monocytes, and the HL-60 and KG-1 myelocytic cell lines. The receptors are few in number, 100 to 500 per cell, and represent a single class of high-affinity binding sites with a KD (dissociation constant) between 15 to 30 pmol/L. In distinction to the binding of GM-CSF to HL-60 and KG-1, Park et al did not detect GM-CSF receptors on U937. In the present study, the effect of GM-CSF on the differentiation of both human peripheral blood monocyte cultures and VD3-stimulated U937 was investigated. We report that VD3 and GM-CSF had a synergistic effect on U937 differentiation as demonstrated by increased OKM1 expression, increased Fc-mediated erythrophagocytosis, and increased chemotaxis and chemokinesis, whereas GM-CSF alone increased OKM1 expression and both chemokinesis and chemotaxis in monocyte cultures.
cells/mL was induced to differentiate by incubating the cells with 10^{-4} mol/L VD3 (provided by Dr Milan Uskokovic, Roche Laboratories, Nutley, NJ). Recombinant human GM-CSF (Genzyme, Boston) or recombinant human G-CSF (Amgen, Thousand Oaks, CA) was added to monocyte or U937 cultures at 1 to 1,000 U/mL, and the cells were evaluated after 48 to 72 hours. The biologic activity of the recombinant G-CSF was confirmed by a proliferation assay using the interleukin 3 (IL-3)-dependent NFS-60 murine cell line.59

Enzyme-linked immunoabsorbent microassay. OKM1 antigen expression was quantified by micro-ELISA as described previously.60 Briefly, control or induced U937 were washed, resuspended in phosphate-buffered saline (PBS) and dried at 37°C onto 96-well plates at 2 x 10^5 cells/well. The plates were then blocked for 15 minutes with 5% dry milk in PBS (Carnation Instant Dry Milk) and incubated with a monoclonal antibody against OKM1 antigen for two hours at 37°C. The plates were then washed and incubated with biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA) followed by an avidin-biotinylated peroxidase complex (Vector). The micro-ELISA was developed by using 2,2-azino-di-(3-ethyl-benzthiazol-2'-sulfonate) (Kirkegaard & Perry Laboratories, Gaithersburg, MD) as substrate. The change in optical density at 405 nm was quantitated by using a Dynatech MR 600 microplate reader (Dynatech Laboratories, Inc, Alexandria, VA).

Flow cytometry. Human peripheral blood monocyte and induced U937 cultures were detached from flasks by scraping and incubated in suspension with antiserum against OKM1 for 90 minutes at 4°C in PBS containing 1% fetal calf serum, 0.1% sodium azide, and 100 µg/mL of human IgG. After incubation, the cell suspensions were washed and resuspended in fluorescein isothiocyanate-conjugated, F(ab')2 sheep anti-mouse IgG (Cooper Biomedical, Malvern, PA). The cells were incubated for 60 minutes at 4°C, washed, resuspended in 0.1% paraformaldehyde in PBS to 10^6 cells/mL, and analyzed with an EPICS V flow cytometer (Coulter Electronics, Hialeah, FL). Log fluorescence was captured in 256-channel, forward-angle light scatter–gated histograms. The mean fluorescence intensity of each sample was calculated by using EASY2 software (Coulter).

Chemotaxis. Control and induced U937 cells, 2 x 10^5, or monocytes, 5 x 10^5, were placed in the upper well of a 48-well chemotactic chamber (Neuro Probe, Inc, Cabin John, MD) and separated from the lower chamber using 1-µm pore size, polycyvinylpyrrolidone-free, Nucleopore polycarbonate membrane filter (Neuro Probe). After incubation for two hours at 37°C, the filters were removed, fixed in methanol, stained in eosin and hematoxylin, and mounted on glass plates. Chemotaxis was quantitated by scanning wells with an LKB Ul troScan XL densitometer (LKB, Bromma, Sweden) interfaced with an IBM PC using the 2400 GelScan XL software (LKB) to measure peak heights and to integrate peak areas. Chemokinesis was evaluated in parallel wells by using media alone in the bottom chamber. All samples were performed in triplicate.

Fc-mediated erythrophagocytosis. Chromium (New England Nuclear, Boston)-labeled sheep erythrocytes were incubated with a 1/500 dilution of 7S rabbit anti-sheep erythrocyte sera (Cordis Laboratories, Miami) for 30 minutes at 37°C. After incubation, antibody-coated or noncoated erythrocytes were washed, and 200 µL of a 0.5% erythrocyte suspension was added to 10^6 control or induced U937 cells in 1.5-mL Eppendorf tubes. The cells were incubated for 90 minutes at 37°C, and extracellular and membrane-associated erythrocytes were lysed with three 1-mL aliquots of Tris-buffered NH₄Cl. The cell pellets were lysed with 1% sodium dodecyl sulfate and counted for chromium. Ingestion of antibody-coated, chromium-labeled erythrocytes by human monocyte cultures was assayed by using a similar protocol after detaching the adherent cells with a rubber policeman. Alternatively, chromium-labeled erythrocytes were first incubated with the adherent monocyte cultures and then scraped, washed with Tris-buffered NH₄Cl, lysed, and counted.

RESULTS

OKM1 expression. Increased expression of macrophage cell surface markers has been reported after VD3, γ-interferon–, retinoic acid–, or phorbol ester–induced differentiation of U937. In the present study, OKM1 expression was quantitated by flow cytometry after a 72-hour incubation of U937 with 10^{-4} mol/L VD3, 50 U/mL of GM-CSF, or VD3 plus GM-CSF. Human peripheral blood monocyte cultures were also incubated in the presence or absence of GM-CSF at 50 U/mL for 72 hours and evaluated for changes in OKM1 expression. As demonstrated in Fig 1, there was no significant expression of OKM1 on the surface of the noninduced U937 cells, whereas cells induced with VD3 had significant levels of OKM1. Treatment of U937 cells with GM-CSF alone resulted in only a slight increase in OKM1 levels over noninduced cells. However, the incubation of U937 cells with GM-CSF plus VD3 resulted in a significant increase in OKM1 expression. Both the mean fluorescence intensity and the percent positive cells were increased relative to U937 cells stimulated with VD3 alone. The Fc receptor control (nonshaded curve in each panel) remained relatively constant under all the induction conditions. In distinction to the lack of effect of GM-CSF alone on U937 differentiation, monocytes cultured for 72 hours with GM-CSF had an increase in both the percentage of positive cells and the amount of OKM1 expressed per monocyte relative to parallel control cultures. Incubation of monocyte cultures with VD3 plus GM-CSF did not result in any further increase in OKM1 expression over that observed for monocytes cultured with GM-CSF alone (data not shown). Increased OKM1 expression on U937 cells was observed after 48 hours incubation with VD3 and GM-CSF (Fig 2). Maximal levels of OKM1 were expressed on U937 cells after 72 hours incubation. The increased expression of OKM1 in VD3-stimulated

![Fig 1. Effect of VD3 and GM-CSF on OKM1 expression. U937 (A to D) or peripheral blood monocytes (E, F) were cultured for 72 hours as nonstimulated (A, E), VD3-stimulated (B, F), or VD3- and GM-CSF-stimulated (D) cells. All cultures were processed for flow cytometry by using antisera directed against OKM1 (solid curve) and an irrelevant IgG1 protein as the Fc receptor control (nonshaded curve). The first number in each panel represents the mean fluorescence intensity and the second, the percent OKM1-positive cells.](image-url)
Fig 2. Kinetics of OKM1 expression. U937 cultures were incubated with VD3, VD3 plus 50 U/mL GM-CSF, or VD3 plus 50 U/mL G-CSF, and aliquots were removed at 24-hour intervals and evaluated for OKM1 expression by micro-ELISA. Note the approximate twofold increase in OKM1 expression when comparing VD3 plus GM-CSF to VD3 alone or VD3 plus G-CSF. This is a representative experiment, and the mean value of triplicates is presented.

Fig 3. Effect of CSF concentration on OKM1. U937 cultures were incubated with 10^{-8} mol/L VD3 plus G-CSF or GM-CSF at concentrations between 0.01 and 50 U/mL for 72 hours. OKM1 expression was quantitated on triplicates by micro-ELISA. The percent increase in OKM1 expression in the CSF-stimulated cultures is compared with the increase in OKM1 observed for cells stimulated with VD3 alone, which is expressed as 100%. —□—VD3 + GM-CSF; —▲—VD3 + G-CSF.

U937 cells were specific for GM-CSF; incubation of parallel VD3-stimulated cells with G-CSF for 72 hours did not increase OKM1 levels (Fig 3). Incubation of U937 cells with VD3 and 1 U/mL of GM-CSF increased the OKM1 levels compared with cultures induced only with VD3. Further increases in OKM1 expression were observed by increasing the concentration of GM-CSF to 50 U/mL. Increasing the concentration of GM-CSF to 1,000 U/mL resulted in no further increase in OKM1 (data not shown).

Chemotaxis. The incubation of U937 cells with VD3 and GM-CSF also resulted in significant increases in both chemotaxis and chemokinesis. Control U937 or U937 stimulated with either VD3 or GM-CSF alone displayed only slight random or directional movement towards f-Met-Leu-Phe. However, U937 cells stimulated with VD3 plus GM-CSF demonstrated significant chemotaxis with maximal movement detected at 10^{-7} mol/L f-Met-Leu-Phe (Fig 4).

Fig 4. Stimulation of U937 chemotaxis by VD3 and GM-CSF. U937 cells were cultured with 50 U/mL of GM-CSF for 72 hours in the presence (■) or absence (○) of VD3. The cells were subsequently washed, and 2 x 10^{6} cells in 50 μL were placed in the upper wells of a Neuro Probe chemotaxis chamber. Media or f-Met-Leu-Phe from 10^{-8} to 10^{-11} mol/L was placed in the lower wells. After incubation for 90 minutes at 37°C, the Nucleapore membranes separating the two chambers were removed, stained, and scanned by using the LKB Ultrascan XL densitometer. Peak heights for each well in triplicate were determined and expressed as relative absorbance units. This is a representative experiment of four; brackets indicate SEM.

U937 cells stimulated with VD3 and GM-CSF also had increased movement toward zymosan-activated serum (data not shown). Human peripheral blood monocyte cultures also demonstrate an increase in both chemokinesis and chemotaxis after incubation for 72 hours with GM-CSF at 100 or 500 U/mL (Fig 5). The dose response to f-Met-Leu-Phe also appears broader in the GM-CSF-stimulated cultures. Freshly isolated monocytes in contrast do not demonstrate any increase in chemotaxis when incubated with comparable concentrations of GM-CSF for two hours. The relative absorbance units of control monocytes migrating towards 10^{-8} mol/L f-Met-Leu-Phe was 0.112 (SEM 0.007) and 0.109 (SEM 0.006) for monocytes incubated for two hours with 100 U/mL of GM-CSF.

Fc-mediated erythrophagocytosis. U937 cells induced to differentiate with VD3 and GM-CSF also had an increased level of Fc-mediated erythrophagocytosis (Table I). This increase in Fc-mediated erythrophagocytosis...
occurred without any increase in the ingestion of non-antibody-coated erythrocytes. The nondifferentiated U937 cells failed to demonstrate Fe-mediated erythrophagocytosis. U937 cultures stimulated with VD3 alone had an approximately threefold increase in erythrophagocytosis \((P < .01)\), whereas cells stimulated with VD3 and GM-CSF had a sixfold increase \((P < .02)\). The increased ingestion of antibody-coated erythrocytes observed for U937 cells incubated with GM-CSF alone was not statistically significant. In distinction to the stimulatory effect of VD3 plus GM-CSF on U937 Fe-mediated erythrophagocytosis, incubation of monocyte cultures with 100 U/mL of GM-CSF for 72 hours failed to demonstrate any further increases in erythrophagocytosis over the control cultures. The addition of VD3 resulted in a consistent decrease in Fe-mediated erythrophagocytosis. The lack of effect of GM-CSF on monocyte erythrophagocytosis was observed in additional experiments with both freshly isolated and three- to five-day-old monocyte cultures incubated with GM-CSF \((50 \text{ to } 500 \text{ U/mL})\) for two hours (freshly isolated) or for the entire culture period (data not shown).

### DISCUSSION

GM-CSF has been demonstrated to support the formation of granulocyte, macrophage, and eosinophil colonies from human bone marrow cultures and to increase many neutrophil, monocyte, and eosinophil effector functions including phagocytosis and ADCC.\(^5\) Treatment of HL-60 cells with GM-CSF has been reported to both increase\(^6\) and have no effect\(^1\) on the percentage of esterase-positive cells. GM-CSF also increased colony formation by HL-60 and KG-1 myeloid lines.\(^7\) GM-CSF has also been reported to increase the expression of OKM1/Mol antigen and f-Met-Leu-Phe receptors on neutrophils\(^12\) and to increase Leu-M3 and Leu-7 on several small-cell lung cancer cell lines.\(^32\)

In the present study VD3 and GM-CSF had a synergistic effect on the differentiation of U937. Increases in OKM1 antigen expression, chemotaxis and chemokinesis, and Fe receptor-mediated erythrophagocytosis were greater than that observed for U937 cells induced with VD3 or GM-CSF alone. Similar increases in OKM1 antigen expression, chemokinesis, and chemotaxis were also observed with human monocyte cultures incubated with GM-CSF alone, although no further increases in Fe-mediated erythrophagocytosis were observed with these same cultures. It is not clear why monocyte cultures did not demonstrate increased Fe-mediated erythrophagocytosis. It is possible that these cultures were already ingesting maximal amounts of antibody-coated erythrocytes under the assay conditions used. Scatchard analysis using soluble, well-characterized iodinated aggregates of immunoglobulin or immune complexes could be used to address this point further. The increase in Fe-mediated erythrophagocytosis detected in U937 cells is, however, consistent with the increased phagocytosis of opsonized yeast and bacteria observed for neutrophils and eosinophils incubated with GM-CSF.\(^13\) The increased chemotaxis and chemokinesis observed in GM-CSF-stimulated monocytes or U937 cells stimulated with VD3 and GM-CSF for 72 hours, however, is in contrast to previous reports on the inhibitory effects of GM-CSF on neutrophil chemokinesis.\(^17\)

The synergistic effect of VD3 and low concentrations of GM-CSF on U937 differentiation appeared to be specific for GM-CSF because these cells remained nonresponsive to G-CSF. In contrast, G-CSF has been reported to bind to cells from acute myeloblastic leukemias, chronic myeloid leukemias, and acute promyelocytic leukemias and to induce the differentiation of the human promyelocytic leukemic cell line HL-60.\(^13\)\(^33\) Significant binding of murine G-CSF to the murine myelomonocytic leukemic cell line WEHI-3B(D+) and to the murine macrophage cell line J774 has also been reported.\(^4\) Whether G-CSF binds to noninduced or VD3-stimulated U937 cells remains to be determined.

The nondifferentiated U937 cells remained essentially nonresponsive to GM-CSF, presumably due to the absence of GM-CSF cell surface receptors.\(^3\) The increased differentiation of U937 cells observed by coincubating with VD3 and GM-CSF suggests that VD3 stimulates the appearance of functional GM-CSF receptors. GM-CSF receptor-bearing U937 cells could then bind and internalize the receptor-ligand complex, as has been reported for murine GM-CSF on the WEHI-3B(D+) cell line,\(^28\) thereby augmenting macrophage effector functions. Therefore, upregulation of GM-CSF receptors may represent an important event during myelomonocytic cell differentiation. To this extent, Gasson et al have reported an increase in the number of GM-CSF receptors on HL-60 cells from 50 to 250 receptors per cell after dimethyl sulfoxide–induced differentiation.\(^26\) The physiological signals associated with upregulation or induction of GM-CSF receptors in vivo remains to be determined.

The ability to augment macrophage differentiation by coincubating human macrophage cell lines with distinct stimulatory agents has been previously observed.\(^3\)\(^5\) Incubation of U937 cells with VD3 plus γ-interferon–containing supernatants resulted in increased OKM1 expression relative to cells stimulated with VD3 alone.\(^1\) Increased expression of OKM1 and other markers was also observed in HL-60 and U937 cells when coincubated with tumor necrosis factor and γ-interferon.\(^3\)
In addition to activated T cells, synthesis of GM-CSF has been demonstrated in IL-1–stimulated endothelial cells, endotoxin-stimulated murine macrophage cell lines, and elicited murine peritoneal macrophages. These observations together with those of the present study suggest that the elaboration of GM-CSF and other stimulatory factors by activated macrophages, endothelial cells, and T lymphocytes results in myeloid cell differentiation after the induction of GM-CSF receptors on receptor-negative cells.

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