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INDUCTION OF MONOCYTIC DIFFERENTIATION BY CALCITRIOL (1,25-DIHYDROXYVITAMIN D_3) IN THE HUMAN PROMYELOCYTIC LEUKEMIC CELL LINE (HL-60) IN SERUM-FREE MEDIUM*

YOUNGSOOK LEE, BRIAN E. DUNLAP and WILLIAM S. MELLON†
School of Pharmacy, University of Wisconsin, Madison, WI 53706, U.S.A.

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Abstract—The effect of calcitriol on the induction of differentiation in human promyelocytic leukemic cell line (HL-60) cultured in serum-free chemically defined medium (SFM) was investigated. The utilization of SFM containing RPMI-1640 basal medium supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), sodium selenite (5 ng/ml), and bovine serum albumin (0.5 mg/ml) allowed a more precise examination of the cellular/molecular mechanism of calcitriol's action in HL-60 cell differentiation without interference of components present in serum. HL-60 cells grown in SFM were induced to differentiate into monocytes/macrophages by calcitriol as indicated by induction of differentiation-associated biological and biochemical parameters: chemiluminescent (CL) responsiveness, lysozyme activity, nonspecific esterase, expression of cell surface antigens, and reduced proliferation. The exposure of HL-60 cells in SFM to calcitriol (from 10^{-10} to 10^{-8} M) resulted in dose-dependent induction of these parameters, which was similar to those obtained with cells grown in 10% fetal calf serum containing medium (10% SCM). However, calcitriol was 5-fold more potent for HL-60 cells cultured in SFM than those cultured in 10% SCM as indicated by shifts in dose–response curves for induction of CL responsiveness and lysozyme activity. The effect of calcitriol on the proliferation and acquisition of several monocyte-associated cell surface antigens was also more sensitive for HL-60 cells cultured in SFM than for cells grown in 10% SCM. We characterized and quantitated calcitriol receptors in HL-60 cells cultured in SFM in comparison to those in 10% SCM after exposing intact cells to radiolabeled calcitriol. Cells cultured in either SFM or 10% SCM exhibited calcitriol receptors that migrated at 3.4*S* as a single peak on sucrose gradients and elicited inherent DNA binding ability. There was essentially no difference in the apparent dissociation constants (K_d) nor in the number of calcitriol binding sites per HL-60 cell, that is $\sim 6.0 \times 10^{-11}$ M and ~3000 binding sites/cell respectively. It is concluded that culturing HL-60 cells in SFM results in full expression of calcitriol-induced phenotypic changes excluding the possibility that such changes result from the indirect effect of calcitriol mediated by identified and/or unidentified components present in serum.

The biologically active metabolite of calciol, 1,25-dihydroxyvitamin D_3 (calcitriol), has been shown to play a major role in mineral metabolism at such target tissues as intestine, bone and kidney (see Refs. 1 and 2 for reviews). The mode of calcitriol's action within target tissues appears to be similar to that of steroid hormones [3], in that calcitriol first binds to a specific, high affinity receptor which consequently induces the expression of mineral-regulating proteins [4–6]. Receptors for calcitriol have been identified not only in the "classical target tissues" [7–9], but in other normal [10, 11] and neoplastic tissues [12–14] including cells of the hematolymphopoietic tissues [15–18]. Furthermore, calcitriol receptors are present in cell lines with monocytic and myeloid characteristics [15–18]. In these neoplastic and monocytic/myeloid calcitriol receptor positive cells, calcitriol has several actions. Calcitriol has been shown to suppress the proliferation of malignant melanoma cells [19]. Moreover, calcitriol promotes monocytic-like differentiation in murine myeloid leukemia cells (M1) [20], human promyelocytic leukemia cells (HL-60) [21–23] and monoblastic U-937 cells [24]. These findings together with the demonstration that calcitriol mediates the differentiation of HL-60 cells into active bone resorbing macrophages [25] engenders the distinct possibility that calcitriol may play a central role in the maturational processing of hematolymphopoietic, immune and bone resorbing cells.

The availability of several clonal calcitriol receptor positive cell lines should enhance our ability to ascertain the role of calcitriol in cellular differentiation. In particular, HL-60 cells may provide a suitable model system for these investigations, especially for describing the involvement and regulation of the calcitriol receptor in the cellular differentiation process. Among various inducers such as vitamins, tumor promoters, highly polar compounds, and anticancer agents [26–30], calcitriol has been shown to be the most potent inducer of HL-60 differentiation [21]. The potencies of several calciol analogs have been correlated roughly with their specificities and affinities for the calcitriol cytosolic receptor in HL-60 cells [15]. However, due to the presence of serum in the culture medium during these studies, absolute

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† To whom all correspondence should be addressed.

potencies for calciol metabolites could not be assessed accurately. While the evidence to date suggests that calcitriol directly mediates HL-60 cellular differentiation, it is conceivable that components contained within the serum-supplemented medium may be responsible, in part, for modifying the calcitriol response. Aside from the known components contained in serum that could induce or modify HL-60 differentiation (i.e. retinoids, interferons, calciol metabolites and other hormones), growth factors and other unknown components may obfuscate calcitriol's mode of action [31, 32]. Serum also contains a number of proteins, including vitamin D-binding proteins which could alter calcitriol pharmacokinetics. In addition, serum can vary widely in its composition from batch to batch. Thus, the interpretation of the experimental results can be complicated not only by the presence of serum, but by the variation in the makeup of its components. Therefore, elimination of undefined substances, hormones and differentiating factors present in serum from the culture medium should facilitate studies assessing the action of calcitriol on growth and differentiation of these human promyelocytic leukemia cells.

In the present study, we report that HL-60 cells proliferated continuously in a serum-free chemically defined medium (SFM) which was similar in composition to the one described by Breitman *et al.* [33]. Moreover, cells grown in SFM retained the capacity to differentiate to mature monocytes when incubated with calcitriol. Lastly, analysis of occupied calcitriol receptors in HL-60 cells cultured in SFM and serum-containing medium (SCM) indicated that serum-free conditions did not alter the physical/chemical properties of occupied calcitriol receptors.

MATERIALS AND METHODS

Materials. Calcitriol, radiolabeled with tritium at the 26,27-methyl groups ([^{3}H]calcitriol, 180 Ci/mmol), was obtained from the Radiochemical Center (Amersham, Buckinghamshire, UK). Nonradioactive calcitriol, (24*R*)-hydroxycalcidiol, and (1*S*)-hydroxycalciol were gifts from Dr. M. Uskokovic of Hoffmann-LaRoche, Inc. (Nutley, NJ), and calcidiol from Dr. Paul W. O'Connell of the Upjohn Co. (Kalamazoo, MI). Phorbol 12-myristate-13-acetate (PMA) was purchased from the Sigma Chemical Co. (St. Louis, MO), hydroxylapatite from Bio-Rad Laboratories (Richmond, CA), and Dulbecco's phosphate-buffered saline (DPBS) from GIBCO (Grand Island, NY). Dimethyl sulfoxide (DMSO) was purchased from the Fisher Scientific Co. (Fair Lawn, NJ), and 3-aminophthalhydrazide (luminol) from the Aldrich Chemical Co., Inc. (Milwaukee, WI). All other reagents were of analytical grade.

Buffers. The buffers used throughout these experiments included: 50 mM Tris–HCl, pH 7.5, 1.5 mM EDTA, 2 mM dithiothreitol (DTT) (TED); 50 mM Tris–HCl, pH 7.5, 1.5 mM EDTA, 5 mM DTT, 10 mM sodium molybdate (TEDM); TEDM with various concentrations of KCl (e.g. TEDMK-0.03 = 0.03 M KCl in TEDM); 10 mM Tris–HCl, pH 7.5, 0.5 mM DTT, 0.03 M KCl (TDK); and 10 mM Tris–HCl, pH 7.5, 0.5 mM DTT, 0.1% Triton X-100 (v/v) (TDT).

Cell culture. The human promyelocytic leukemia cells, HL-60, isolated from the peripheral blood leukocytes of a patient with acute promyelocytic leukemia [34] were obained from American Type Tissue Culture (Bethesda, MD) and were maintained in RPMI-1640 medium buffered with 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) (Sigma Chemical Co.) containing 100 units/ml penicillin and 0.5 μg/ml fungizone. This basal medium was supplemented with either heat-inactivated (56° for 30 min) 10% (v/v) fetal calf serum (Sterile System, Inc., Logan, UT), designated as 10% SCM, or with a defined supplement of insulin from bovine pancreas (5 μg/ml), human transferrin (5 μg/ml), sodium selenite (5 ng/ml), and bovine serum albumin (BSA) (fatty acid free) (0.5 mg/ml) (Sigma Chemical Co.), designated as serum-free medium (SFM). Cells were grown at 37° in a humidified 95% air–5% CO_2 atmosphere and subcultured with 4- to 5-day intervals at a seeding density of 2×10^5 cells/ml. Cells subcultured in SFM were maintained in this defined nutrient medium for several weeks involving at least 5 to 10 passages before experiments were initiated. Cell proliferation was assessed by counting cells in a hemocytometer, and cell viability was determined by trypan blue dye exclusion.

Calciol metabolite treatment. HL-60 cells were seeded in multi-well plates with either 10% SCM or SFM. Except where indicated, cells were treated for 3 days in duplicate wells with various concentrations of calciol metabolites, calcitriol, (24*R*)-hydroxycalcidiol, (1*S*)-hydroxycalciol and calcidiol, dissolved in ethanol. Control cultures were incubated with an appropriate amount of vehicle (ethanol). The final ethanol concentration was less than 0.1% (v/v).

Chemiluminescence assay. Determination of chemiluminescent (CL) responsiveness was carried out as described elsewhere [35, 36]. Briefly, calciol metabolite treated HL-60 cells and control cells (10^6) were added to 7.0-ml glass scintillation vials. After cells were washed once with 2.0 ml of DPBS and once with 1.0 ml of Krebs Ringer phosphate buffer, pH 7.4, containing 0.2% BSA and 0.2% glucose (KRP), they were resuspended in 1.0 ml of KRP buffer followed by the addition of 10 μl luminol and 10 μl PMA at a final concentration of 10^{-8} M and 10 μg/ml respectively. CL measurements were made serially in a Packard Tri-Carb ambient-temperature liquid scintillation counter with use of the tritium window setting and with the coincident circuit turned off.

Lysozyme activity. Lysozyme activity was determined as described elsewhere [37], with a slight modification. Briefly, the HL-60 cells (1×10^6) were centrifuged at 500 *g* for 5 min to obtain the supernatant fraction after treatment with various calciol metabolites. The enzyme reaction was initiated by adding 0.1-ml aliquots of supernatant fraction to 0.9 ml of 0.066 M phosphate buffer, pH 6.24, containing 0.15 mg of dry *Micrococcus lysodiekticus* (Sigma) at 30°. The turbidity of the reaction mixture was measured by spectrophotometry at 450 nm for 20 min.

Nonspecific esterase activity. Cytochemical assays for nonspecific esterase activity (α-naphthylacetate esterase) were performed with a commercially available kit (Sigma technical bulletin, No. 90). The enzyme activity can be detected by the formation of highly colored deposits at the sites of enzyme activity. The results are expressed as the percentage of cells with black granulation regardless of the intensity of the coloration. A field of at least 200 cells was counted for each determination.

Indirect immunofluorescence. To measure the binding to cells of monoclonal antibodies (Ab's) which react primarily with monocytes and macrophages, indirect immunofluorescence assays were performed using a fluorescence-activated cell sorter (FACS IV; Becton Dickinson, Mountain View, CA) as described by the manufacturer's direction (Coulter Immunology, Hialeah, FL). Briefly, HL-60 cells (1×10^6) were placed into 12 × 75 mm Falcon tubes and washed with ice-cold DPBS two times. The reaction of monoclonal Ab with cells was initiated by adding 200 μl of monoclonal Ab solutions, Anti-Leu-M3 (Beckton Dickinson Monoclonal Center, Inc., Mountain View, CA) or 63D3 (Bethesda Research Laboratories, Inc., Gaithersburg, MD), to cells and incubating them at 4° for 30 min. After washing two times with 1 ml of wash medium [PBS containing 2% (v/v) fetal calf serum and 0.01% (w/v) sodium azide], 200 μl of fluorescein isothiocyanate conjugated second Ab (goat antiserum to mouse immunoglobulin, Coulter Immunology) was added to the cell pellet and incubated at 4° for 30 min. After the third wash with resuspension medium [PBS containing 0.01% (w/v) sodium azide], cells were then resuspended in 1 ml of wash medium and subjected to FACS IV analysis. The negative control (only second Ab was added to the control cells) was employed for the determination of background.

Preparation of calcitriol receptors. HL-60 cells (50×10^6) cultured in either 10% SCM or SFM were rinsed and resuspended in 2 ml of serum-free RPMI-1640 medium and SFM respectively. After cells were labeled with 1 nM [^{3}H]calcitriol in the presence or absence of 200-fold excess of calcitriol for 1.5 hr at 37° in a humidified 95% air–5% CO_2 atmosphere, cells were rinsed two times in DPBS, followed by centrifugation at 500 *g* for 5 min. The following manipulations were carried out between 0 and 4°. After cells were resuspended and incubated for 20 min in 2 ml of ice-cold TED buffer, cells were homogenized using a Tissuemizer type SDT (Tekmar Co., Cincinnati, OH) for 3 sec at a speed setting of 50. The homogenate was centrifuged for 10 min at 800 *g*, and the pellet was designated as a crude nuclear fraction. The crude nuclear pellet was incubated with TEDMK-0.3 for 30 min with intermittent vortexing followed by centrifugation at 105,000 *g* for 45 min in a Beckman L5-50B ultracentrifuge using a type Ti-50 rotor (Beckman Instruments, Palo Alto, CA). The supernatant fraction was utilized as the source of [^{3}H]calcitriol labeled nuclear receptor for physical/chemical characterization.

Sucrose density gradient analysis. Aliquots of [^{3}H]calcitriol receptor were applied to linear sucrose density gradients (4–20%) in TEDMK-0.3 to observe sedimentation characteristics of calcitriol–receptor complexes, as described elsewhere [38]. Gradients were centrifuged for 18 hr at 235,000 *g* in a Beckman ultracentrifuge (model L5-50B) using a SW-60 rotor between 0 and 4° and subsequently fractionated as described elsewhere [38]. To each 0.1-ml fraction was added 4.0 ml of a scintillation fluid [39] and the radioactivity was determined by liquid scintillation spectrometry in a Packard Tri-Carb Spectrometer (model Prias PLD) with an efficiency for tritium of 40%. Quench correction was determined by the use of automatic external standardization.

Binding studies. HL-60 cells grown in either 10% SCM or SFM were rinsed and resuspended at a density of 10×10^6 cells per 1 ml of serum-free RPMI-1640 medium and SFM respectively. After HL-60 cells were labeled with various concentrations of [^{3}H]calcitriol in the presence or absence of a 200-fold excess of calcitriol for 1 hr at 37° in a humidified 95% air–5% CO_2 atmosphere, cells were rinsed two times with DPBS and resuspended in 1 ml of ice-cold TED for 20 min with intermittent vortexing. Receptor-bound [^{3}H]calcitriol was quantitated by an hydroxylapatite (HAP) batch assay [40] with slight modification. Briefly, 0.3 ml of HAP slurry [50% (v/v) in TDK buffer] was added to each incubation tube containing 0.3 ml of [^{3}H]calcitriol labeled receptor. The samples were kept at 0–4° for 15 min with intermittent mixing and centrifuged at 1500 *g* for 5 min. The HAP pellet was washed three times with 1.0 ml of TDT buffer and extracted twice with 1.0-ml portions of chloroform–methanol (1:2, v/v); the supernatant fractions were combined and dried under a stream of air at 35°. To each vial was added 4.0 ml of a scintillation fluid [39], and the radioactivity was quantitated by liquid scintillation spectrometry with an efficiency for tritium of 45%.

DNA-cellulose chromatography. DNA-cellulose chromatography was performed as described elsewhere [41]. Briefly, the [^{3}H]calcitriol receptor was treated with dextran-coated charcoal to remove unbound calcitriol. The labeled nuclear receptor was diluted ten times (v/v) with TEDM to reduce ionic strength (i.e. to a final concentration of KCl equal to 30 mM), and aliquots were applied to a 10 ml DNA-cellulose column previously equilibrated with TEDMK-0.03 buffer. Chromatography was initiated by washing the column with TEDMK-0.03 buffer, followed by a linear KCl gradient (TEDMK-0.03–0.50) for the elution of labeled receptor. Fractions (2.5 ml) were collected, and 1.5-ml aliquots were removed to determine the radioactivity as described above.

Statistical analysis. Where appropriate, statistical comparisons of the means of the independent samples were made using Student's *t*-test.

RESULTS

Effect of calcitriol on HL-60 cell proliferation. In SFM, growth of HL-60 cells continued at a rate approximately 73% of that occurring in 10% SCM (Fig. 1). Subsequently, we observed more similar growth rates (i.e. a range of 80–90% of that occurring in 10% SCM) when cells were seeded in SFM at a density of 4–5 × 10^5 cells/ml (unpublished results).

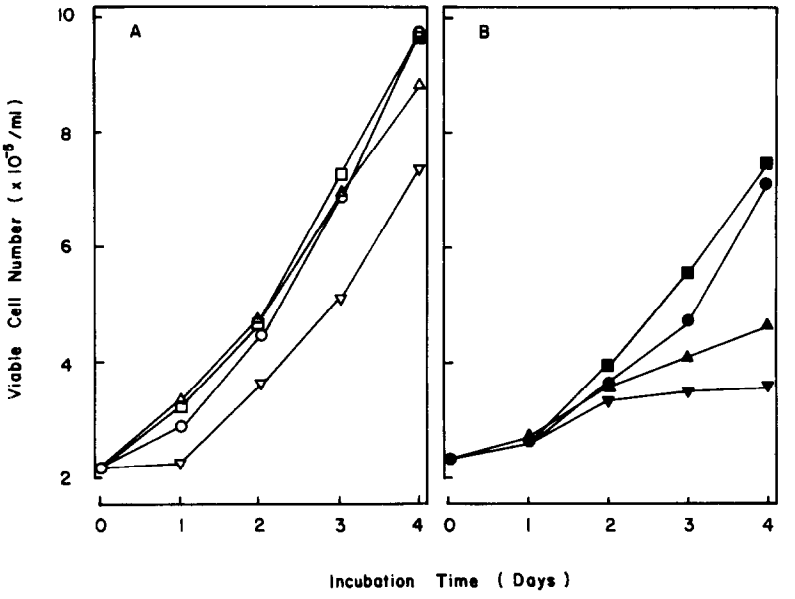

Fig. 1. Effects of calcitriol on cell growth of HL-60 cells cultured in 10% SCM (open symbols) (A) and in SFM (closed symbols) (B). Cells were inoculated at 2×10^5 cells/ml in duplicate wells and incubated for 4 days with 0.1% ethanol (○, ●) (control), or calcitriol 0.2 nM (□, ■), 5.0 nM (△, ▲) or 10.0 nM (▽, ▼). On the days indicated, cells were harvested, and the viable cell number was counted as described in Materials and Methods. Each point is a mean ±SE of duplicate measurements from the average of eight separate experiments.

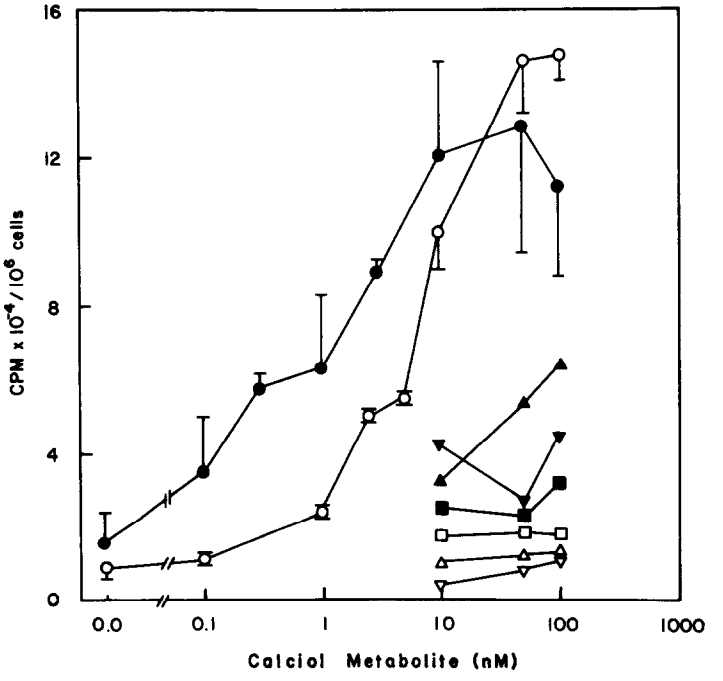

Fig. 2. Effect of calciol metabolites on induction of CL responses in HL-60 cells cultured in either 10% SCM or SFM. Cells (4×10^5/ml) were incubated with various concentrations of calcitriol (○, ●), (1*S*)-hydroxycalciol (△, ▲), (24*R*)-hydroxycalcidiol (▽, ▼), or calcidiol (□, ■) for 3 days. Open symbols and closed symbols represent CL responses in cells cultured in 10% SCM and SFM respectively. CL levels for 10^6 cells were determined 10 min after PMA was aded to the reaction mixture as described in Materials and Methods. Values represent the means ±SE of duplicate determinations from an average of five replications.

Treatment with calcitriol suppressed proliferation of HL-60 cells in a dose- and time-dependent manner. Figure 1 shows a biphasic dose response in which a slight stimulation of growth occurred in cells cultured in SFM with 0.2 nM calcitriol. At a concentration of 10 nM calcitriol, the viable cell number was reduced to 75 and 50% of control on day 4 in 10% SCM and SFM respectively.

Chemiluminescent responsiveness. Chemiluminescence may be viewed as a manifestation of the microbicidal activity of monocytes and granulocytes. To assess the CL responses of HL-60 cells, cells were treated with various concentrations of calciol metabolites for 3 days (preliminary studies indicated that 3-day treatment was the optimum for determination of CL responses). The results in Fig. 2 demonstrate the dose–response relationship for several calciol metabolites and the induction of CL in cells cultured in 10% SCM and SFM. The concentrations of calcitriol that induced 50% of maximal CL were estimated to be 1.6 and 7.8 nM in HL-60 cells grown in SFM and 10% SCM respectively. The maximal level of CL response induced by calcitriol in cells grown in SFM was not significantly different from that in 10% SCM ($P > 0.05$). Next, effects of other metabolites, (1*S*)-hydroxycalciol, (24*R*)-hydroxycalcidiol and calcidiol on the induction of CL were compared in SFM and 10% SCM. Of these metabolites tested, only (1*S*)-hydroxycalciol was capable of inducing significantly higher CL in HL-60 cells, albeit requiring concentrations of several orders of magnitude greater than for calcitriol.

Lysozyme production. Normal macrophage can synthesize and secrete *in vitro* copious amounts of lysozyme which is one of the antibacterial proteins [42], and calcitriol-treated HL-60 cells exhibit much the same characteristics. The dose–response effects of various calciol metabolites on the production of lysozyme are shown in Fig. 3. Preliminary data indicated that 3-day treatment with calcitriol was optimum to measure lysozyme activity. The concentrations of calcitriol that induced 50% maximal lysozyme production were estimated to be 0.9 and 4.4 nM in HL-60 cells grown in SFM and 10% SCM respectively. The maximal lysozyme induction by calcitriol in cells grown in SFM was not significantly different from that in 10% SCM ($P > 0.05$). The order of the potency in inducing lysozyme activity was similar to that in inducing CL. These results, together with those in Fig. 2, indicate that the potencies of calciol metabolites in inducing differentiation were calcitriol $\gg$ (1*S*)-hydroxy calciol > calcidiol $\equiv$ (24*R*)-hydroxycalcidiol in cells grown in SFM.

Nonspecific esterase activity. α-Naphthylacetate esterase is detected primarily in monocytes and is virtually absent in granulocytes. Untreated HL-60 cells exhibited little enzyme activity, whereas treated HL-60 cells in either SFM or 10% SCM became α-naphthylacetate esterase positive in a concentration-dependent manner, with over 50% of cells stained at a concentration of 10 nM calcitriol (Table 1). Albeit, it was apparent by visual examination that the intensity of the color for cells treated with cal-

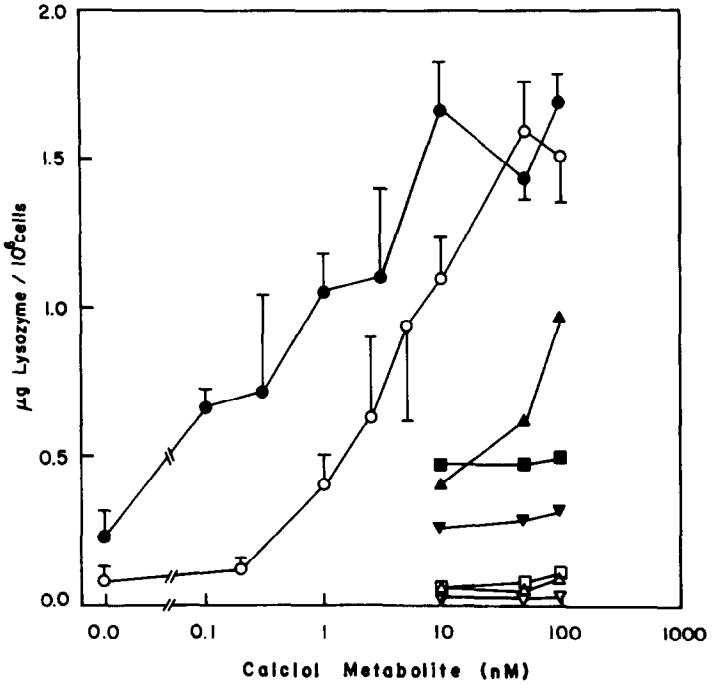

Fig. 3. Induction of extracellular lysozyme by calciol metabolites in HL-60 cells cultured in either 10% SCM or SFM. The cells (4×10^5/ml) were incubated with various concentrations of calcitriol (○,), (1*S*)-hydroxycalciol (△, ▲), (24*R*-hydroxycalcidiol (▽, ▼), or calcidiol (□, ■) for 3 days; open symbols for 10% SCM and closed symbols for SFM. The data represent the means ±SE of triplicate measurements from the average of six replications.

Table 1. Induction of α-naphthylacetate esterase activity in HL-60 cells by calcitriol

Added concentration of calcitriol (nM)	α-Naphthylacetate esterase positive (%) 10% SCM	SFM
0.0	<1	<1
0.2	2	3
1.0	6	9
10.0	57	57
50.0	80	63

Cells (4×10^5/ml) were inoculated in either 10% SCM or SFM and incubated for 3 days in the presence or absence of various concentrations of calcitriol. Data are expressed as the percentage of total cells assayed and represent the mean of four replications with triplicate measurements.

citriol and cultured in SFM was greater than for those in 10% SCM (data not shown).

Indirect immunofluorescence. To detect cell surface specific antigen expression characteristic of mature monocytes and macrophages, indirect immunofluorescence was performed using monoclonal Ab. While untreated HL-60 cells had little reactivity with two monoclonal antibodies tested, monocyte-specific cell surface antigens clearly were detected on calcitriol-treated cells in a dose–response manner (Table 2). Between 60 and 80% of HL-60 cells grown in 10% SCM and SFM, respectively, expressed both Leu-M3 and 63D3 antigens with 20 nM calcitriol treatment. In addition, data in Table 2 reveal that calcitriol exhibited a greater potency for stimulating both Leu-M3 and 63D3 antigens in cells cultured in SFM.

Characterization and quantitation of calcitriol receptor. For the investigation of the mechanism whereby calcitriol induces differentiation in HL-60 cells, a series of experiments for the characterization of calcitriol specific receptor were performed. Since we attempted to remove the unknown factors in serum by using SFM, it was necessary to characterize and quantitate calcitriol receptor in HL-60 cells cultured in SFM and 10% SCM.

The specific uptake of [^{3}H]calcitriol into intact HL-60 cells was measured as described in Materials and Methods. Figure 4 demonstrates the saturable and high affinity specific binding of [^{3}H]calcitriol for intact HL-60 cells. Examination of the specific binding by Scatchard analysis revealed linear plots whose apparent equilibrium dissociation constants were 5.7×10^{-11} M and 6.4×10^{-11} M for HL-60 cells cultured in 10% SCM and SFM respectively. An extrapolation to the abscissa is estimated to be ~3000 calcitriol binding sites per HL-60 cell cultured in either 10% SCM or SFM. Aliquots of [^{3}H]calcitriol receptors, prepared by labeling intact HL-60 cells cultured in 10% SCM or SFM with [^{3}H]calcitriol (see Materials and Methods), were analyzed by sucrose density gradient centrifugation (data not shown). A peak with a sedimentation coefficient of 3.4*S* was found for HL-60 cells cultured in either SFM or 10% SCM. In both cases the peak was completely displaced in the presence of a 200-fold excess of unlabeled calcitriol. To demonstrate and compare the affinity of the calcitriol–receptor complex for DNA, the calcitriol receptor from HL-60 cells cultured in either SFM or 10% SCM was applied to a DNA-cellulose column and eluted with a buffered salt gradient, 0.03 to 0.5 M KCl (data not shown). One peak of [^{3}H]calcitriol binding component in HL-60 cells cultured in either 10% SCM or SFM appeared, which was retained under low ionic strength and eluted at a KCl concentration of 0.18 M.

DISCUSSION

One of the major problems confronting research using cultured cells has been the variability of results obtained from experiment to experiment on a day-by-day basis. It has been reported that 26% of HL-60 cells cultured in 10% fetal calf serum containing medium became phagocytic cells after exposure to

Table 2. Monocyte-specific antigen expression on HL-60 cells by calcitriol: Indirect immunofluorescence with FACS IV analysis*

Added concentration of calcitriol (nM)		Monoclonal Ab: Anti-Leu-M3 MFI†	Anti-Leu-M3 Pbc‡	Anti-63D3 MFI	Anti-63D3 Pbc
0.0	10% SCM	46.6	1.1	43.2	0.0
	SFM	50.2	3.5	44.6	1.72
0.5	10% SCM	46.8	2.8	44.5	2.38
	SFM	64.3	36.4	60.2	35.19
5.0	10% SCM	59.1	28.5	58.3	30.88
	SFM	76.8	60.7	72.5	60.97
20.0	10% SCM	75.6	57.0	72.2	59.27
	SFM	86.9	79.7	81.8	81.19

* On the basis of light scatter properties, dead cells were excluded from fluorescence analysis. Light scatter windows were also chosen to maximize purity of the analyzed populations.

† MFI, normalized mean fluorescence intensity.

‡ Pbc, percentage of cells brighter than negative control fluorescence.

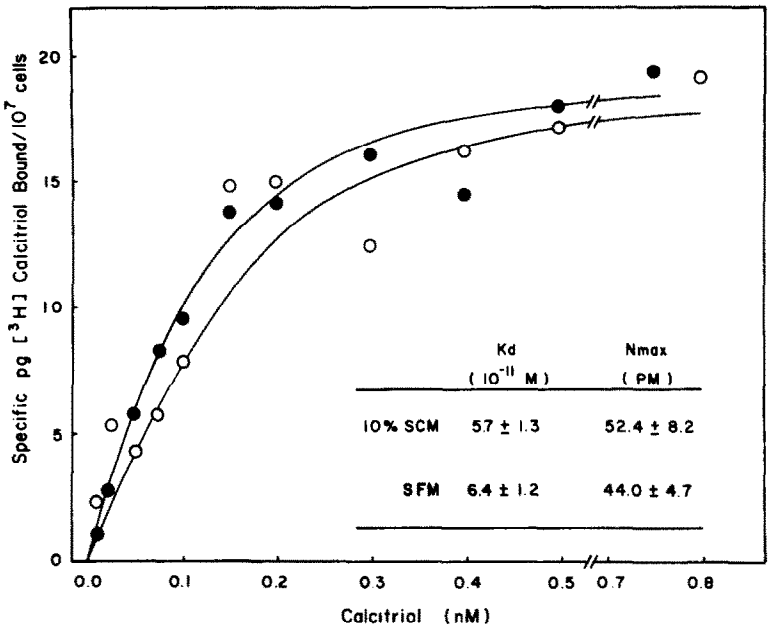

Fig. 4. Determination of the apparent equilibrium dissociation constant (K_d) for [^{3}H]calcitriol binding to receptors in intact HL-60 cells cultured in 10% SCM (○) and in SFM (●). Receptor-bound [^{3}H]calcitriol was quantitated by an HAP batch assay as described in Materials and Methods. Values represent the means of four to six replications with duplicate determinations. The apparent K_d values for specific [^{3}H]calcitriol binding calculated from the slopes of the regression lines were 5.7×10^{-11} and 6.4×10^{-11} for HL-60 cells in 10% SCM and SFM, respectively, while the concentrations of specific binding sites estimated from the abscissa intercepts were ~3000 bindings sites for an HL-60 cell cultured in either 10% SCM or SFM (see inset for Scatchard transformation).

10 nM calcitriol for 3 days [18], while over 50% of HL-60 cells became phagocytic cells under the same culture condition in another laboratory [21]. It has been also shown that 54% of HL-60 cells became nonspecific esterase positive cells with 10 nM calcitriol treatment for 3 days [18] as opposed to about 95% of nonspecific esterase positive cells with the same culture condition [25]. These variabilities may be accounted for, in part, by the variability of the serum in which the cells are grown. Not only does serum contain undefined proteins and factors, but serum can vary widely in its composition from batch to batch.

We have demonstrated that calcitriol caused concentration-dependent maturation of HL-60 cells grown not only in 10% SCM but also in SFM, as evidenced by its ability to decrease cell proliferation, and to induce CL responsiveness and lysozyme production (Figs. 1–3). Treatment of HL-60 cells with calcitriol resulted in a concentration-dependent increase of α-naphthylacetate esterase enzyme activity (Table 1) which has been shown to occur primarily in monocytes and is virtually absent in granulocytes. In addition, HL-60 cells acquired monocyte-specific cell surface antigens in a dose-dependent manner. These data clearly indicate that HL-60 cells grown in SFM were able to differentiate toward the monocyte/macrophage pathway after exposure to calcitriol. Moreover, the data also suggest that calcitriol directly mediates this differentiation process rather than via a factor(s) contained within serum. These results are consistent with the findings of Breitman *et al.* [33] who demonstrated that HL-60 cells cultured in serum-free nutrient medium supplemented with transferrin and insulin could differentiate to mature granulocytes in the presence of DMSO. The doses of calcitriol reported in the literature, which have been shown to induce phenotypic changes in HL-60 cells cultured in 10–20% serum supplemented medium, are generally larger than would be predicted by the equilibrium dissociation constant for its receptor [15, 18, 21, 22, 43]. This incongruence tends to obfuscate the issue as to whether this hormone is important for "normal" hematopoietic maturation. Furthermore, extrapolating these *in vitro* doses to doses that would be clinically effective for treating hematopoietic disorders raises serious concern, given the potential toxicities of calcitriol. We sought to address these issues using a chemically defined medium, not only to assess the dependence of calcitriol-induced differentiation on serum, but also to ascertain the potency of calciol metabolites under conditions that more closely approximated an extracellular environment. In this regard, calcitriol exhibited a 2- to 20-fold increase in potency for inducing several phenotypic changes in HL-60 cells cultured in SFM. Although Table 1 indicates that SFM did not potentiate a non-specific esterase response, this is partially due to our use of the appearance or non-appearance of black granules as the assay end point. Microscopic observation of stained slides indicated a quantitative increase in black granulation in calcitriol-treated cells cultured

in SFM versus those treated in 10% SCM, but non-uniform cell adhesion and wide variation in individual cell staining prevented a reliable quantification of this increase.

The increased potency of calcitriol in SFM is consistent with those results obtained by several other laboratories. Ball *et al.* [43] demonstrated that HL-60 cells express class 1 HLA antigens when cultured in nutrient medium supplemented with serum and not in a chemically defined serum-free medium. Moreover, calcitriol was able to clearly induce these antigens only in the serum-free medium. Additional corroboration of our results is supported by the findings of Amento *et al.* [26]. In the absence of serum, changes in U937 cell morphology and decreases in cell proliferation occurred with lower doses of calcitriol than in those cells cultured in the presence of serum. Thus, the phenotypic changes reported by us and others utilizing chemically defined serum-free media occur at concentrations of the active hormone that are more closely associated with physiological concentrations.

Decreased cellular responsiveness in the presence of serum also has been demonstrated for a wide variety of cell types. It has been reported that serum causes a 5- to 10-fold decrease in the sensitivity of T-lymphoma cells to growth inhibition by cyclosporin A [44]. In some systems, serum has been shown to inhibit the differentiated function of granulosa cells [45–47]. Moreover, progesterone production in response to FSH is higher in porcine granulosa cells cultured in serum-free medium than in those grown in the presence of 10% fetal calf serum [48].

The lower potency of calcitriol in HL-60 cells cultured in 10% SCM could be a consequence of the vitamin D-binding protein in serum which could decrease the free hormone concentration in the culture medium. This view also has been advanced previously by Amento *et al.* [26] for calcitriol-induced differentiation in U937 cells. In contrast to the uptake of calcitriol in SFM, uptake into intact HL-60 cells was reduced greatly in the presence of 10% fetal calf serum (data not shown). These results are consistent with the hypothesis that serum binding proteins may be acting as a reservoir for the hormone. However, it has been suggested previously that serum binding may not be the only cause for decreases in cell sensitivity to a drug or ligand [44]. Therefore, we cannot exclude the possibility that other substances contained in serum could be antagonizing the differentiating effect of calcitriol in HL-60 cells.

The relative order of potency for calcitriol metabolites causing induction of differentiation in HL-60 cells cultured in SFM was calcitriol $\geqslant$ (1*S*)-hydroxycalciol > calcidiol $\cong$ (24*R*)-hydroxycalcidiol. The inability of calcidiol and (24*R*)-hydroxycalcidiol to induce differentiation of HL-60 cells in 10% SCM may be due to the fact that these metabolites have less affinity for the calcitriol receptor [15]. In addition, the vitamin D-binding protein in serum binds calcidiol and (24*R*)-hydroxycalcidiol to a greater degree than calcitriol [49, 50], thereby lowering the concentration of these free calciol metabolites available for entry into the cells. The specificity of these calciol metabolites tested in the present study in inducing differentiation was well correlated with the specificity of their association with the putative calcitriol receptor [15]. The existence of a positive correlation between calcitriol-induced differentiation and the occurrence of occupied calcitriol receptors has been reported previously in HL-60 cells [18]. Likewise, the functional defect of the calcitriol resistant HL-60 clones appears to be correlated with the reduced amount of the specific calcitriol receptor [51]. These results are supportive of the hypothesis that calcitriol-induced differentiation in HL-60 cells is receptor mediated [15, 18, 51], although the mechanism of calcitriol-induced differentiation of HL-60 cells remains unknown.

Since calcitriol was shown to be a more potent cellular differentiating agent when cells were cultured in SFM in comparison to SCM, it was important to examine whether the calcitriol receptor had undergone changes in physical/chemical characteristics. Such structural or chemical differences might be related to the observed differences in hormone potency. For example, other cultured cells grown in serum-free media show enhanced numbers of β-adrenergic receptors or changes in regulation of epidermal growth factor receptors [44, 52] compared to cells grown in serum-containing medium. Culturing HL-60 cells in serum-free conditions does not seem to alter the physical/chemical characteristics of the calcitriol binding protein qualitatively or quantitatively. Similar sedimentation patterns were observed for the [^{3}H]calcitriol receptor in HL-60 cells grown in SFM and SCM. The 3.4*S* sedimentation coefficient is similar to that reported previously for mammalian calcitriol receptors [1, 7]. The inherent DNA-cellulose binding ability of the calcitriol receptor also was not affected, as indicated by the similar elution patterns of DNA cellulose chromatography. The equilibrium dissociation constant of calcitriol receptor and the number of binding sites per cell remained unchanged in HL-60 cells cultured in SFM. Thus, it is unlikely that physical/chemical alterations in calcitriol receptors are responsible for the increased potency of calcitriol in HL-60 cells grown in SFM. Moreover, the significant alterations in the physical nature of the receptor might be unlikely from the findings that growth of HL-60 cells in SFM increases the potency but not the efficacy of calcitriol.

In the present study, HL-60 cells that had proliferated in SFM were still capable of differentiating toward the monocyte/macrophage pathway after exposure to calcitriol. Furthermore, employing SFM for HL-60 cell culture did not alter the physical/chemical characteristics of the calcitriol binding protein qualitatively. Many cell types can now be cultured in completely defined media [32], and the utilization of these systems in the study of the molecular mechanism of hormone action may be quite fruitful. Thus, further studies can now be pursued utilizing a more "physiological milieu" without the variation introduced by the use of serum.

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