1,25-Dihydroxyvitamin D_3 Production and Vitamin D3 Receptor Expression Are Developmentally Regulated During Differentiation of Human Monocytes Into Macrophages

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It has been well established that human mononuclear phagocytes have the capacity to produce 1,25-dihydroxyvitamin D_3 [1,25(OH)$_3D_3$] and express the vitamin D receptor (VDR). However, 1α-hydroxylase activity and VDR receptor expression during differentiation of monocytes (MO) into mature macrophages (MAC) have not been previously examined. The in vitro maturation of blood MO can serve as a model for the in vivo transformation of immature blood MO into MAC. Here, when cultured in the presence of serum, MO undergo characteristic changes in morphology, antigenic phenotype, and functional activity consistent with their differentiation into MAC. We serially measured 1,25(OH)$_2D_3$ and 24,25-dihydroxyvitamin D_3 [24,25(OH)$_2D_3$] synthesis, specific [^{3}H]-1,25(OH)$_2D_3$ binding, and VDR mRNA levels during in vitro maturation of MO into MAC and correlated these functions with maturation-associated changes in the phenotype (MAX.1 and CD71) and secretory repertoire (interleukin-1β [IL-1β], neopterin) of the cells. MO showed only little conversion of 25-(OH)D_3 into 1,25(OH)$_2D_3$ (1.4 ± 0.4 pmol/10^6 cells/6 h, n = 5) that increased gradually during maturation into MAC at day 8 of culture (5.3 ± 4.3 pmol/10^6 cells/6 h, n = 5). Interferon-γ (IFN-γ) increased baseline 1,25(OH)$_2D_3$-synthesis approximately twofold during all phases of differentiation. The time course of increased 1,25(OH)$_2D_3$-synthesis correlated with enhanced secretion of neopterin and expression of MAX.1 and CD71. The addition of exogenous 1,25(OH)$_2D_3$ did not influence constitutive 1,25-(OH)$_2D_3$ synthesis, but IFN-γ–stimulated production was suppressed to baseline levels. Exogenous 1,25(OH)$_2D_3$ also stimulated 24,25(OH)$_2D_3$ synthesis in freshly isolated MO (from 1.0 ± 0.8 pmol/6 h to 5.6 ± 0.9 pmol), whereas matured MAC showed no 24,25(OH)$_2D_3$ synthesis. Furthermore, we examined the expression of the VDR during the differentiation process. VDR mRNA and protein were constitutively expressed in MO, whereas VDR was downregulated in mature MAC on both the mRNA and protein levels. Homologous upregulation of VDR protein by 1,25(OH)$_2D_3$ occurred in MO and, to a lesser degree, in MAC. In contrast, VDR mRNA concentrations were not influenced by 1,25(OH)$_2D_3$. Taken together, our results show that MO into MAC differentiation in vitro is associated with (1) an enhanced capacity to synthesize 1,25-(OH)$_2D_3$, (2) a loss of 24,25(OH)$_2D_3$-synthesizing activity, and (3) a decrease in the expression of VDR mRNA and protein. Because 1,25(OH)$_2D_3$ was shown to induce differentiation of MO into MAC, our data suggest an autoregulatory mechanism of MO/MAC generation by 1,25(OH)$_2D_3$.

1,25-DIHYDROXYVITAMIN D_3 [1,25(OH)$_2D_3$] is a principal regulator of bone and mineral metabolism. In addition, this hormone has multiple effects on cells of the immune system.[1] 1,25(OH)$_2D_3$ induces differentiation of several human cell lines of the monocytic lineage[2,3] and of normal human hematopoietic cells.[4,5] The hormone modulates various functions of monocytes (MO), such as antigen presentation, secretion of prostaglandin E_2, and hydrogen peroxide,[6,7,8] and influences the activity of T and B lymphocytes.[9,10]

Most of the biologic actions of 1,25(OH)$_2D_3$ are mediated through a hormone-receptor complex in a manner analogous with the mechanism of action for classical steroid hormones.[11] Vitamin D_3 receptors (VDR) are present in various normal and leukemic hematopoietic cells,[12] eg, in MO and activated B and T lymphocytes. In contrast, the VDR is absent from normal resting B and T lymphocytes.[18] The cDNA for the human 1,25(OH)$_2D_3$ receptor shows sequence homology to the thyroid hormone receptor and other steroid hormone receptors.[14]

Furthermore, evidence exists that cells of the mononuclear phagocyte system (MPS) can metabolize 25(OH)D_3 into 1,25(OH)$_2D_3$. Normal bone marrow and alveolar macrophages synthesize 1,25(OH)$_2D_3$ after stimulation with interferon-γ (IFN-γ) or lipopolysaccharide (LPS).[15,16] In sarcoidosis, tuberculosis, and some cases of malignant lymphoma, the deregulated extrarenal 1,25(OH)$_2D_3$ production seems to play a crucial role in the generation of hypercalcemia.[17-19] Tissue macrophages (MAC) are thought to be the source of 1,25(OH)$_2D_3$ in these diseases. Some evidence exists that 24,25-dihydroxyvitamin D_3 [24,25(OH)$_2D_3$] can also be produced by MO/MAC.[15]

The terminal differentiation step from circulating blood MO into MAC is accompanied by characteristic changes in morphology, functional activity, and antigen expression.[20,21] This differentiation process can also be followed in vitro when blood MO are cultured in the presence of serum.[22] Using this in vitro model of MAC maturation, we studied the developmental regulation of vitamin D metabolism and the regulation of VDR expression in human MO/MAC.

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Submitted June 8, 1992; accepted March 19, 1993.

Supported by Deutsche Forschungsgemeinschaft. A. S. was supported by Humboldt-Foundation.

*Presented at the 33rd Annual Meeting of the American Society of Hematology, December 6-10, 1991, Denver, CO (*Blood *78:107a, 1991 [abstr, suppl 1]).*

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0006-4971/93/8204-0108$3.00/0

MATERIALS AND METHODS

Cell separation and culture. Peripheral blood mononuclear cells (MNC) were obtained by leukapheresis of healthy donors, followed by density gradient centrifugation over Ficoll/Hypaque. MO were isolated from MNC by countercurrent elutriation (J6M-E Beckmann centrifuge; Beckmann, Munich, Germany) using a large-volume chamber (50 mL), a JE-5 rotor at 2,500 rpm, and a flow rate of 110 mL/min in Hanks' Balanced Salt Solution supplemented with 2% human albumin. Elutriated MO were more than 90% pure as determined by morphology and antigenic phenotype. Purified MO were cultured on Teflon foils (Biofolie 25; Heraeus, Hanau, Germany) for up to 14 days at a cell density of 10^6 cells/mL in RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 5 $\times$ 10^{-5} mol/L mercaptoethanol, polyvitamins, antibiotics, pyruvate, nonessential amino acids, and 5% pooled human AB-group serum. After the indicated time period, cells were harvested, washed twice, counted by trypan blue exclusion, and cultivated for another 24 hours in 6-well microtiterplates or petridishes (both from Falcon/Becton Dickinson, Heidelberg, Germany) as described below.

Hydroxylase assay. MO or MO-derived MAC were seeded into 6-well microtiterplates at 10^6 cells/well/mL in RPMI 1640 without serum. Cells were grown in the presence of 500 U/mL IFN-γ (Bioferon, Laupheim, Germany), 10^{-8} mol/L $1,25(OH)_2D_3$ (kindly provided by Hoffmann-La Roche, Basel, Switzerland), IFN-γ, and $1,25(OH)_2D_3$ or in vehicle for 18 hours. Then, 50 nmol/L 25-(26,27-methyl-^{3}H)$(OH)D_3$ was added (165 Ci/mmol; Amersham, Braunschweig, Germany) for another 6 hours. In experiments performed in the presence of $1,25(OH)_2D_3$, every well was washed 3 times with RPMI 1640 before adding 25-(^{3}H)$(OH)D_3$. Control experiments were performed using only medium and 25-(^{3}H)$(OH)D_3$ without cells. 1α-Hydroxylase activity was not detected in control incubations. The reaction was terminated by adding 1 mL methanol, and cells were detached from the plastic surface with a cell scraper. Cells and supernatants were harvested and stored at -20°C until use. All incubations were performed in duplicate or triplicate.

Production of MO/MAC supernatants. MO or MO-derived MAC were seeded at 10^6 cells/2 mL into 6-well microtiterplates in RPMI 1640 with 2% pooled human AB-group serum. Cells were stimulated with 100 ng/mL LPS Salmonella abortus equi (kindly provided by Chris Galanos; Max-Planck Institut für Immunbiologie, Freiburg, Germany) or IFN-γ (500 U/mL) for 24 hours. Controls were performed without stimulus. Supernatants were harvested, filtered through 0.22 μm filters, and stored at -20°C.

Detection of interleukin-1β (IL-1β) and neopterin. IL-1β was measured by commercially available sandwich enzyme-linked immunosorbent assay (Biochrom, Laupheim, Germany) and neopterin by radioimmunoassay (IBL, Hamburg, Germany).

RNA-extraction and Northern analysis. Either MO or MO-derived MAC obtained after a culture period of 7 days in Teflon bags were seeded into plastic dishes at a density of 10^6 cells/mL and cultured in the presence or absence of $1,25(OH)_2D_3$ (10^{-8} mol/L) or IFN-γ (200 U/mL), respectively. After 4 hours the supernatant was discarded, cells were lysed directly in the dishes with guanidiumthyocyanate solution and RNA extraction was performed according to Chomzynski and Sacchi.[23] Six micrograms of total RNA was run in 1% agarose-formaldehyde gels and transferred to nylon membranes (Nytran, Schleicher and Schüll, Dassel, Germany).

VDR mRNA was detected by hybridization of the membranes with a full-length human VDR cDNA probe (kindly provided by W. Pike, Houston, TX)[14] labeled with (^{32}P)d-cytidine triphosphate (3,000 Ci/mmol; Amersham, Buckinghamshire, UK) by the random-prime method with a commercially available kit (Amersham). As a control, glycerolaldehyde-3-phosphate-dehydrogenase (GAP-DH) mRNA was detected by hybridization with an oligonucleotide probe complementary to bp 1101 through 1187 of the published sequence.[24] This probe was labeled with T4 polynucleotide kinase (Boehringer Mannheim, Mannheim, Germany) and γ(^{32}P)adenosine triphosphate (5,000 Ci/mmol; Amersham). Hybridization conditions were 500 mmol/L sodiumphosphate pH 7.2, 7% sodium dodecyl sulfate (SDS), 1 mmol/L EDTA, 150 μg/mL tRNA (adapted from Church and Gilbert[25]) at 65°C overnight. Washing conditions were 3 $\times$ 30 minutes 0.1$\times$ SSC, 1% SDS at 52°C. The different probes were hybridized to the same membranes after stripping of the previous probes at 0.1$\times$ SSC, 1% SDS, 70°C for 3 hours.

X-ray films (Kodak XAR; Eastman Kodak Company, Rochester, New York) were exposed to the filters for 10 days (VDR) or overnight (GAP-DH) with an intensifying screen. The intensity of the radioactive signals was determined by laser densitometry (Pharmacia, Uppsala, Sweden) of the X-ray films.

Phenotype analysis. MAC were attached to alcian-blue–coated slides and prefixed with 0.05% glutaraldehyde on ice. They were incubated with the following mouse monoclonal antibodies (MoAb): anti-gp65 (MAX.1, from our laboratory) and anti-CD71 (OKT9, Ortho Diagnostics, Neckargmünd, Germany). A peroxidase-anti-peroxidase (PAP) technique was applied followed by postfixation with OsO_4.[26]

Analysis of 25(OH)D_3 metabolism. Cells and medium were combined for analysis of 25(OH)D_3 metabolism. Lipids were extracted by the procedure of Bligh and Dyer.[27] After evaporation of the chloroform under a stream of nitrogen, the samples were taken up in the appropriate solvent, and the radioactive metabolites were separated by high performance liquid chromatography (HPLC) on a liquid-chromatograph (Waters, Frankfurt, Germany). Radioactive 25(OH)D_3-metabolite peaks were localized by measuring the tritium content of successive 1-minute fractions by liquid scintillation measurements. The tritium peaks were compared with the elution positions of chemically synthesized radioinert vitamin D_3 metabolites that were included in each HPLC run to serve as internal standards. Their migration was determined by UV absorbance measurements at 254 nm. Conversion of 25(OH)D_3 was analyzed with a Radial PAK μPorasil cartridge (Waters) that was eluted over 22 minutes with a 4% to 60% nonlinear gradient of isopropanol in n-hexane (flow-rate 2 mL/min). On this system, 25(OH)D_3 eluted after 5 minutes, 24,25-dihydroxyvitamin D_3 ($24,25(OH)_2D_3$) after 12 minutes, and $1,25(OH)_2D_3$ after 17 minutes. One replicate of each incubation was consecutively chromatographed on hexane-based straight-phase HPLC and on reverse-phase HPLC. Reverse-phase HPLC was performed with a μBondapak C18 steel column (Waters), 0.39 $\times$ 30 cm, eluted with water/methanol (15:85, vol/vol), at a flow rate of 1 mL/min. Thirty-two fractions were collected from this system. In all experiments, the elution position of the metabolite from MO/MAC matched the elution position of chemically synthesized $1,25(OH)_2D_3$ on the two HPLC systems.

Measurement of 1,25(OH)$_2$$D_3$ binding capacity. $1,25(OH)_2D_3$ binding capacity was determined by a modification of a previously described method.[28] Briefly, cultured cells were scraped in ice-cold TEDMo (10 mmol/L Tris HCl, 1.5 mmol/L EDTA, 1 mmol/L DTT, 10 mmol/L sodium molybdate, pH 7.4, 4°C)-phenylmethylsulphonyl flouride (PMSF)/aprotinin (PMSF and aprotinin both from Serva, Heidelberg, Germany) buffer and were then homogenized in TEDMo-PMSF/aprotinin buffer (40% wt/vol) with a Polytron homogenizer. The homogenates were centrifuged (5,000g for 10 minutes) to yield the cytosol fraction and the nuclear pellet. A crude chromatin fraction was prepared resuspending the pellet two times in the above buffer with 0.5% Triton X-100 (Sigma, München, Germany) followed by centrifugation (10,000g for 10 minutes). The pellet was washed with TEDMo buffer without Triton X-100 followed by centrifugation (10,000g for 10 minutes). The resulting crude chromatin pellet was resuspended in TEDMo-PMSF/aprotinin buffer, extensively vortexed, and briefly ultrasoni-

cated for further analysis. Aliquots of the chromatin fraction and the cytosol fraction were removed for protein measurement. Aliquots of the chromatin and the cytosol preparations, respectively, were incubated with 3 nmol/L [^{3}H]-1,25(OH)$_2$D$_3$ for total binding and 200× excess unlabeled hormone for unspecific binding. The assay tubes were vortexed and incubated for 18 hours at 4°C. Bound activity was separated from free activity by batchwise treatment with hydroxyapatite (Sigma) as described previously.[29] Because of limited availability of cells, Scatchard saturation analysis could not be performed. Data are presented as combined-specific [^{3}H]-1,25(OH)$_2$D$_3$ binding capacity from chromatin and cytosol fractions.

RESULTS

25(OH)D$_3$ metabolism at various stages of differentiation. Freshly isolated MO produced small amounts of [^{3}H]-1,25(OH)$_2$D$_3$ (1.4 ± 0.4 pmol/10^6 cells/6 h, n = 5). During cultivation of cells, 1,25(OH)$_2$D$_3$ production increased gradually and typically reached a peak between days 6 and 8 of the culture period. Taken together, 1,25(OH)$_2$D$_3$ production on day 4 (2.7 ± 1.3, n = 4) and day 8 (5.3 ± 4.3, n = 5) by unstimulated cells was significantly ($P < .05$) higher than hormone production by freshly isolated MO. Average 1,25(OH)$_2$D$_3$ production by unstimulated MAC on day 14 (1.2 ± 1.1, n = 5) was comparable to that on day 0. An HPLC chromatogram of [^{3}H]-25(OH)D$_3$ metabolism by day 8 MAC is shown in Fig 1. Further monitoring of 1α-hydroxylase activity up to day 14 showed a decrease in 1,25(OH)$_2$D$_3$ synthesis with increasing culture time. A time course of 1,25(OH)$_2$D$_3$ production during differentiation of MO into MAC is shown in Fig 2. The similarity between 1α-hydroxylase activity (Fig 2A) and the time course of secretion of neopterin (Fig 2B, a marker of mature MAC) is evident. IL-1β, a cytokine that is predominantly secreted by freshly isolated MO, has an inverse correlation to the 1α-hydroxylase activity (Fig 2B). There is also a relationship between the increase of maturation-associated antigens (MAX.1 and transferrin receptor/CD71) and 1α-hydroxylase activity (Fig 2C). Activation of MO/MAC by IFN-γ stimulated 1,25(OH)$_2$D$_3$ production at all time points tested except in freshly isolated MO (Fig 2A). On day 8, there was an approximately twofold increase in 1,25(OH)$_2$D$_3$ synthesis. The time course of 1,25(OH)$_2$D$_3$ production by IFN-γ–treated MO/MAC closely resembled the time course in untreated cells. An increase until day 6 through 8 was followed by a gradual decrease until day 14 (Fig 2A). IFN-γ significantly ($P < .05$) stimulated 1,25(OH)$_2$D$_3$ synthesis on day 4 (6.4 ± 1.4, n = 4) and on day 8 (10.4 ± 7.3, n = 4). On day 14, there was no longer a statistically significant stimulation of 1,25(OH)$_2$D$_3$ synthesis by IFN-γ.

The effect of exogenous 1,25(OH)$_2$D$_3$ on the regulation of the 1α-hydroxylase activity was assessed in further experiments by incubating cells for 18 hours in the presence of 10 nmol/L 1,25(OH)$_2$D$_3$. As summarized in Fig 3, 1,25-(OH)$_2$D$_3$ did not influence 1α-hydroxylase activity in untreated cells at all stages of differentiation (days 0, 4, 8, and 14). In contrast, IFN-γ–activated MAC were sensitive to 1,25(OH)$_2$D$_3$ regulation. The IFN-γ–stimulated increment in hormone synthesis was blocked by exogenous 1,25-(OH)$_2$D$_3$ (Fig 3).

Constitutive 24,25(OH)$_2$D$_3$ production varied somewhat between cell preparations (1 ± 0.8 pmol/10^6 cells/6 h) and was found in 3 of 5 experiments. The baseline 24-hydroxylase activity in MO decreased upon maturation of cells and ceased until day 4. Because renal 24,25(OH)$_2$D$_3$ production is stimulated by 1,25(OH)$_2$D$_3$, we looked for a similar behavior in MO/MAC. The conversion of 25(OH)D$_3$ into 24,25(OH)$_2$D$_3$ during differentiation of MO into MAC after incubation with 1,25(OH)$_2$D$_3$ or 1,25(OH)$_2$D$_3$ plus IFN-γ is shown in Fig 4. A pronounced stimulation of 24,25(OH)$_2$D$_3$ synthesis after exposure to 1,25(OH)$_2$D$_3$ occurred on day 0 (5.6 ± 0.9 pmol/10^6 cells/6 h, n = 2). On day 4, inducible 24-hydroxylase activity decreased markedly; on day 8, the

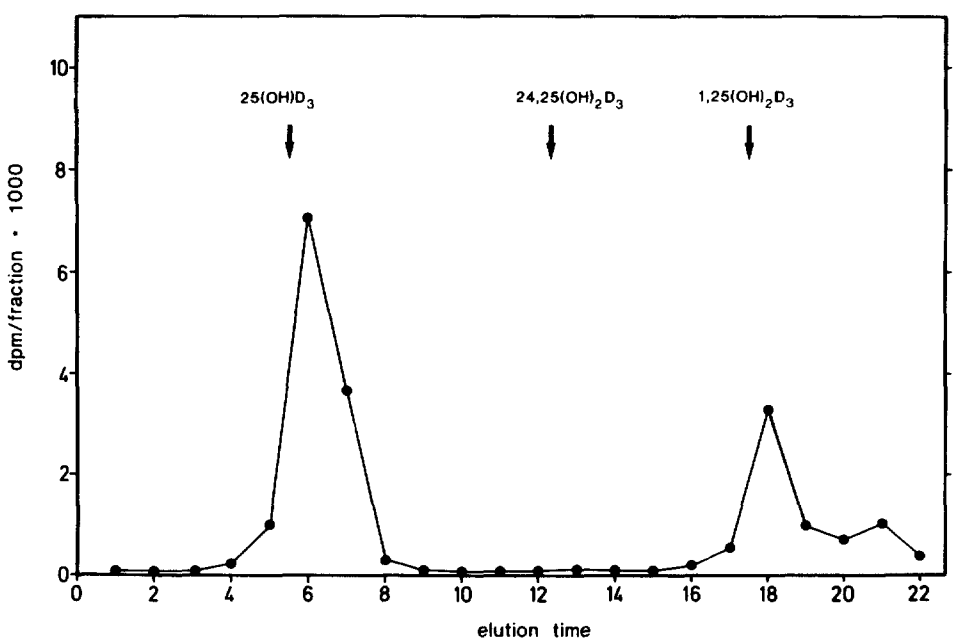

Fig 1. HPLC profile of [^{3}H]-25(OH)D$_3$ metabolism by MO-derived MAC, cultured for 8 days. Cells were incubated for 6 hours with 50 nmol/L 25(OH)([^{3}H])D$_3$. Lipid extracts of cells and media were analyzed by HPLC using a Radial PAK μPorasil cartridge eluted over 22 minutes with a 5% to 60% gradient of isopropanol in n-hexane (flow rate, 2 mL/min). Tritium contents of consecutive 2 mL fractions are shown. The arrows indicate the elution position of authentic vitamin D metabolites.

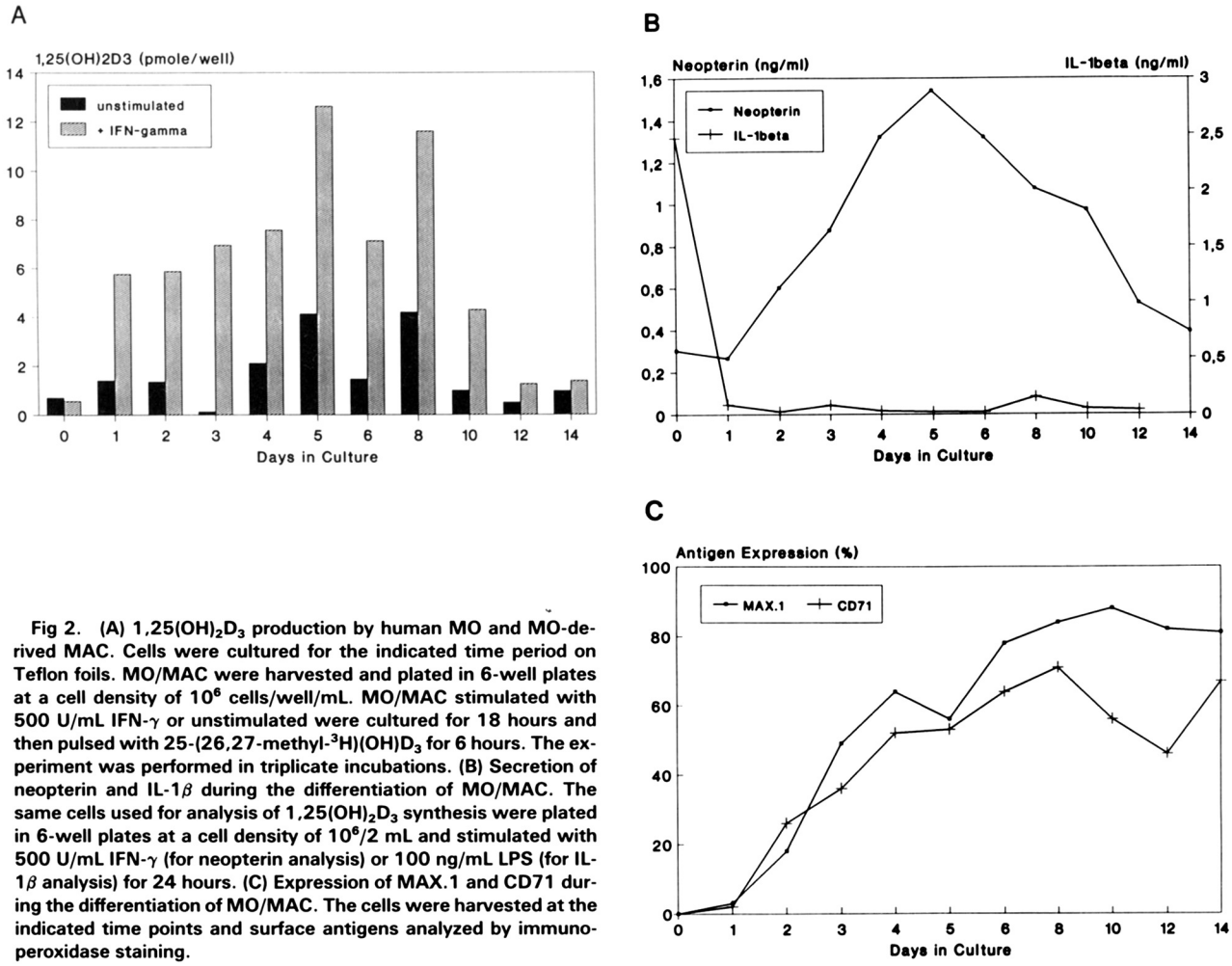

Fig 2. (A) $1,25(OH)_2D_3$ production by human MO and MO-derived MAC. Cells were cultured for the indicated time period on Teflon foils. MO/MAC were harvested and plated in 6-well plates at a cell density of 10^6 cells/well/mL. MO/MAC stimulated with 500 U/mL IFN-γ or unstimulated were cultured for 18 hours and then pulsed with 25-(26,27-methyl-^{3}H)$(OH)D_3$ for 6 hours. The experiment was performed in triplicate incubations. (B) Secretion of neopterin and IL-1β during the differentiation of MO/MAC. The same cells used for analysis of $1,25(OH)_2D_3$ synthesis were plated in 6-well plates at a cell density of 10^6/2 mL and stimulated with 500 U/mL IFN-γ (for neopterin analysis) or 100 ng/mL LPS (for IL-1β analysis) for 24 hours. (C) Expression of MAX.1 and CD71 during the differentiation of MO/MAC. The cells were harvested at the indicated time points and surface antigens analyzed by immunoperoxidase staining.

$24,25(OH)_2D_3$ hydroxylase could no longer be stimulated by $1,25(OH)_2D_3$ (Fig 4). IFN-γ had no clear effect on the 24-hydroxylase. Baseline $24,25(OH)_2D_3$ production by MO on day 0 was similar in the absence or presence of IFN-γ. As compared with $1,25(OH)_2D_3$ alone, the addition of $1,25(OH)_2D_3$ plus IFN-γ resulted in a slight reduction of $24,25(OH)_2D_3$ synthesis (Fig 4).

MAC between days 4 and 8 inconsistently produced $25(OH)(^3H)D_3$ metabolites that were more polar than $1,25(OH)_2D_3$ (Fig 1). The synthesis of the polar material was not related to IFN-γ exposure. This material was not further characterized.

VDR expression at various stages of differentiation. The expression of VDR mRNA in MO and MO-derived MAC was studied in further experiments. Total RNA was isolated either on day 0 or day 7. Figure 5 shows the Northern blots for the VDR compared with the expression of GAP-DH in MO/MAC. MO show a strong, constitutive expression of the VDR mRNA, which is markedly downregulated in MO-derived MAC on day 7. Exposure to $1,25(OH)_2D_3$ or IFN-γ resulted in a slight reduction of VDR mRNA in both MO and MAC (Fig 5). These changes were less pronounced in two other experiments and were not statistically significant.

VDR protein concentrations, as measured by [^{3}H]-$1,25(OH)_2D_3$ binding capacity, are summarized in Table 1. In parallel with VDR mRNA concentrations, VDR protein concentrations in MO were approximately twofold higher than in MAC. In contrast to VDR mRNA data, incubation with $1,25(OH)_2D_3$ caused significant upregulation of [^{3}H]-$1,25(OH)_2D_3$ binding in either cell type. However, the increase of VDR protein was more pronounced in MO.

DISCUSSION

The terminal differentiation process of MO into mature MAC is a crucial event in the development of fully competent mononuclear phagocytes. A similar process occurs in vitro when blood MO are cultured in the presence of serum.[22] MO-derived MAC show a typical MAC morphology and functional activity in terms of high TNF-α and neopterin release and low secretion of IL-1β and IL-6.[30-32] In addition the expression of specific maturation-associated antigens can be detected during in vitro differentiation.[21,33,34] The results of this study show that the capacity of MO/MAC to synthesize $1,25(OH)_2D_3$ and to express the VDR is regulated in a reciprocal fashion during differentiation of cells.

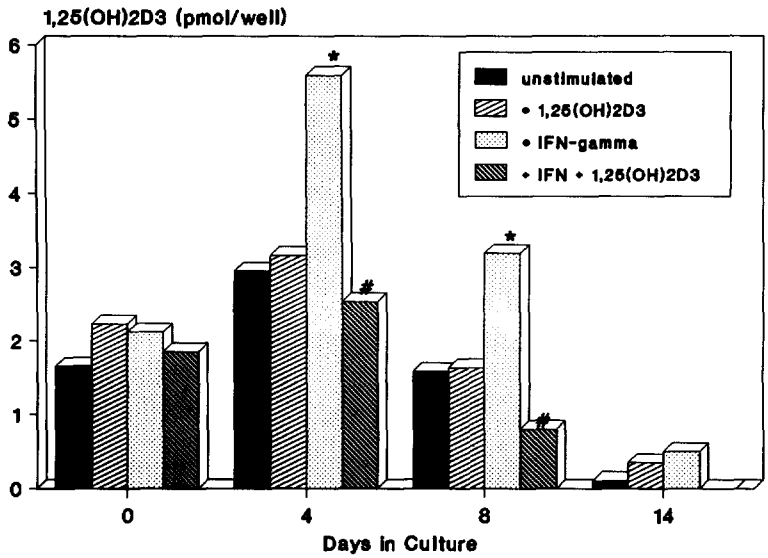

Fig 3. Effect of $1,25(OH)_2D_3$ (10^{-8} mol/L) on hydroxylase-activity in MO/MAC. Cells were cultured for the indicated time period, harvested, and plated in 6-well plates at a cell density of 10^6 cells/well/mL. MO/MAC were stimulated with 500 U/mL IFN-γ, 10^{-8} mol/L $1,25(OH)_2D_3$, or IFN-γ plus $1,25(OH)_2D_3$ for 18 hours. Then cells were washed 3 times and pulsed for another 6 hours with 25-(^{3}H)$(OH)D_3$. Results represent the means of two experiments, each performed in duplicate incubations. ($\star P < .05$ *v* unstimulated, $\#P < .05$ *v* IFN-γ).

Although freshly isolated MO produced relatively low amounts of $1,25(OH)_2D_3$, basal 1-α-hydroxylase activity increased rapidly during the differentiation process of MO into MAC. These changes were accompanied by a characteristic increase of maturation-associated antigens (MAX.1 and CD71) and release of neopterin, whereas IL-1β secretion decreased. These results suggest that the capacity to synthesize $1,25(OH)_2D_3$ is acquired by MO during the differentiation process into MAC, whereas less mature cells have no or little 1-α-hydroxylase activity. Accordingly, immature human cell lines of the monocytic lineage (HL-60 and U937) showed no $1,25(OH)_2D_3$ synthesis,[35] whereas mature pulmonary alveolar MAC (PAM) and bone marrow-derived MAC (BMM) had the ability to convert $25(OH)D_3$ into $1,25(OH)_2D_3$.[15] In our system, $1,25(OH)_2D_3$ production decreased after 8 to 10 days of culture. The reason for this is not apparent, but similar results were found with PAM and BMM.[15]

IFN-γ augmented $1,25(OH)_2D_3$ synthesis by MO/MAC at all time points tested. These results showed that, in addition to the stage of differentiation, the degree of activation is a second determinant of 1α-hydroxylase activity. The responsiveness of 1α-hydroxylase to IFN-γ did not vary in an apparent fashion during differentiation.

In the kidney, there is a feedback regulation of $1,25(OH)_2D_3$ production by exogenous or endogenous $1,25(OH)_2D_3$. Corresponding results with cultured renal epithelial cells showed a downregulation of $1,25(OH)_2D_3$ synthesis and an increase in $24,25(OH)_2D_3$ synthesis.[36] Therefore, we examined the action of exogenous $1,25(OH)_2D_3$ on 1-α-hydroxylase activity in MO/MAC. In contrast to renal cells, basal $1,25(OH)_2D_3$ production was not altered by $1,25(OH)_2D_3$ in MO/MAC. Previous studies with normal and sarcoid PAM and BMM[15,37] also showed little or no inhibition of hormone synthesis by exogenous $1,25(OH)_2D_3$. Dusso et al[38] recently showed that $1,25(OH)_2D_3$ produc-

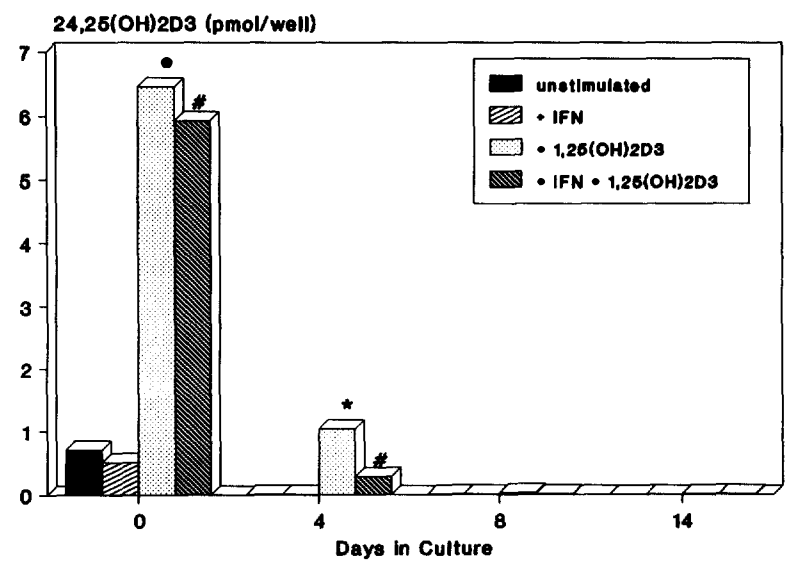

Fig 4. $24,25(OH)_2D_3$-synthesis by MO/MAC. Cells were cultivated for the indicated time period, harvested, and plated in 6-well plates. MO/MAC were stimulated with 10^{-8} mol/L $1,25(OH)_2D_3$, 500 U/mL IFN-γ, $1,25(OH)_2D_3$ plus IFN-γ, or without any stimulus for 18 hours. Then cells were washed and pulsed for another 6 hours with 25-(^{3}H)$(OH)D_3$. Results represent the means of two experiments, each performed in duplicate incubations. ($\star P < .05$ *v* unstimulated, $\#P < .05$ *v* IFN-γ).

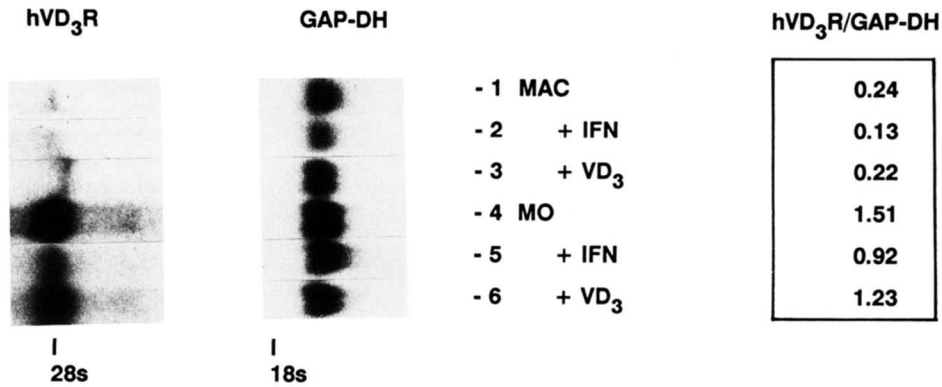

Fig 5. Expression of VDR mRNA in human MO (day 0) and MAC (day 8). Freshly isolated MO or MO-derived MAC were seeded into plastic dishes and cultured in the presence of 500 U/mL IFN-γ, 10^{-8} mol/L $1,25(OH)_2D_3$, or without any stimulus. After 4 hours, RNA was extracted and Northern analysis performed according to Materials and Methods. The VDR/GAP-DH ratios obtained by densitometric screening are shown next to the respective lanes.

tion was inhibited by exogenous $1,25(OH)_2D_3$. The reason for the discrepancy between their findings and our study is not clear.

In contrast to the constitutive $1,25(OH)_2D_3$ synthesis, the IFN-γ augmented production of $1,25(OH)_2D_3$ in MO/MAC was suppressed to baseline levels by exogenous $1,25(OH)_2D_3$. Whereas $24,25(OH)_2D_3$ synthesis could never be induced in MAC, $24,25(OH)_2D_3$ production was regularly detected in freshly isolated blood MO upon the addition of $1,25(OH)_2D_3$. Obviously, the capacity to synthesize $24,25(OH)_2D_3$ was rapidly lost during the differentiation of MO into MAC. Accordingly, others have found $24,25(OH)_2D_3$ synthesis after exposure to $1,25(OH)_2D_3$ in freshly isolated MO[38] and in the monocytic cell line HL-60,[35] whereas mature MAC (PAM, BMM) had no 24-hydroxylase activity after short-term exposure to $1,25(OH)_2D_3$.[15] In contrast, Hayes et al[39] reported spontaneous $24,25(OH)_2D_3$ synthesis in PAM of a patient with rhabdomyosarcoma, but the MAC of this patient might not have been normal.

Most of the actions of $1,25(OH)_2D_3$ are thought to be mediated through intracellular receptors. Therefore, we looked for the expression of VDR during the differentiation of MO into MAC. MO showed a strong constitutive expression of VDR mRNA and VDR protein, whereas VDR was down-regulated in mature MAC on both levels. Homologous upregulation of VDR-protein, which had been shown for various cell types,[40-43] also occurred in cultured MO and MAC. However, in quantitative terms, responsiveness to $1,25(OH)_2D_3$ was lower in MAC than in MO. Homologous upregulation of VDR is likely to have occurred on a post-transcriptional level, because VDR mRNA concentrations were not affected by $1,25(OH)_2D_3$. These data are in agreement with previous data on HL-60 promyelocytic cells and other hematopoietic cells types.[12,42] Taken together, VDR was downregulated during differentiation of MO to MAC. Responsiveness to $1,25(OH)_2D_3$ was, to some degree, preserved in matured MAC, because VDR protein was upregulated after incubation with $1,25(OH)_2D_3$. Because $25(OH)D_3$24-hydroxylase activity could no longer be induced in MAC, differential behavior of $1,25(OH)_2D_3$-responsive genes with respect to exposure of $1,25(OH)_2D_3$ must be discussed.

Table 1. [^{3}H]-$1,25(OH)_2D_3$ Binding Capacity During In Vitro Differentiation of MO to MAC

	[^{3}H]-$1,25(OH)_2D_3$ Binding (fmol/mg protein)
MO	336 ± 57*
MO + $1,25(OH)_2D_3$	554 ± 115†
MO + IFN-γ	270
MAC	170 ± 32
MAC + $1,25(OH)_2D_3$	240 ± 59‡
MAC + IFN-γ	154

Data are the means ± SEM from three experiments (except IFN-γ treatment, for which data are from one experiment).

* $P < .03$ *v* MAC.

† $P < .03$ *v* MO.

‡ $P < .05$ *v* MAC (Mann-Whitney U-test for paired data).

In summary, we show that the capacity of MO/MAC to convert $25(OH)D_3$ to its metabolites $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ and the expression of VDR are dependent on the stage of maturation of the cells. Whereas $24,25(OH)_2D_3$ is mostly produced by freshly isolated MO, which lose this capacity upon differentiation into MAC, the secretion of $1,25(OH)_2D_3$ is upregulated during the maturation process of MO into MAC. These changes are accompanied by a reduction in the constitutive expression of the VDR. Therefore, we conclude that MO (and more immature cells of the monocytic lineage) are the main target for $1,25(OH)_2D_3$,[4,5] because these cells strongly express the VDR. During the differentiation into MAC, there is a downregulation of the VDR, but these cells are potent $1,25(OH)_2D_3$ producers. Taken together, our results suggest an autoregulatory mechanism in which MAC recruit cells of their own lineage from the pool of circulating blood MO through the production of $1,25(OH)_2D_3$. The role of $24,25(OH)_2D_3$ in this system needs to be clarified through further investigation.

ACKNOWLEDGMENT

We thank Helga Ortlepp and Ute Ackermann for excellent technical assistance.

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