RAPID COMMUNICATION

Induction of Human Monocyte to Macrophage Maturation In Vitro by 1,25-Dihydroxyvitamin D_3

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Cells of the mononuclear phagocyte system arise from circulating blood monocytes (MO) that undergo further maturation on leaving the vasculature and migration into the various tissues and body cavities. This terminal differentiation step is also observed in vitro when blood MO are cultured in the presence of serum. Yet, the inducing signals present in serum are not defined. We have established primary cultures from elutriation-purified blood MO and found that the active metabolite of vitamin D_3 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) could induce maturation of MO to macrophages (MAC) in the absence of any serum proteins. Cells were cultured for 7 days with AB-group serum or 1,25$(OH)_2D_3$, respectively, and MO maturation analyzed by morphology, functional activity, and the expression of lineage-restricted maturation-associated antigens (MAX.1, MAX.3). At an optimal concentration of 10^{-8} mol/L, 1,25$(OH)_2D_3$ promoted the development of fully differentiated MAC whose phenotype and functional competence in terms of cytokine release (tumor necrosis factor α, interleukin-6, fibronectin, and lysozyme) was comparable with MAC grown in serum. In conclusion, our data may add to the immunoregulatory potential of 1,25$(OH)_2D_3$, which may play an essential role in the ontogeny of the mononuclear phagocyte system.

CIRCULATING BLOOD monocytes (MO) provide a common precursor pool which gives rise to the heterogenous family of cells that constitute the human mononuclear phagocyte system (MPS).[1] The terminal differentiation of blood MO to mature macrophages (MAC) can also be followed in vitro.[2-5] Here, when cultured in the presence of serum MO undergo a characteristic change in morphology, cytochemistry, and function, a process considered to be similar to the differentiation of MO in vivo. MO maturation in vitro is associated with the expression of specific antigens not found on blood MO but present on macrophages.[6-8] Their analysis is used to more objectively define maturation in primary cultures of human MO.[9,10]

No consistent knowledge presently exists on the regulation of MO to MAC transformation. Serum needs to be present in MO cultures for successful maturation into MAC, neither known hematopoietins nor other yet defined cytokines are able to replace serum. Only macrophage colony-stimulating factor (M-CSF) might be of importance as a competence and survival factor for MO in vitro[11] (Brugger W, Kreutz M, Andreesen R: Macrophage colony-stimulating factor is required for monocyte survival and acts as a cofactor for their terminal differentiation to macrophages in vitro. *J Leukoc Biol*, in press 1991).

The major biologically active metabolite of vitamin D_3, 1,25-dihydroxyvitamin D_3-(1,25$(OH)_2D_3$), has found increasing attention since functional receptors have been found to be ubiquitous in tissue distribution.[12,13] Beside the classical role of 1,25 $(OH)_2$ D_3 in mineral homeostasis this molecule was shown to be an immunoregulatory hormone.[14,15] In addition, several studies using tumor cell lines[16-18] as well as cultures of normal bone marrow precursors[19] have shown 1,25$(OH)_2D_3$ to induce cell differentiation toward the macrophage lineage. There is also preliminary evidence that 1,25$(OH)_2D_3$ supports the serum-induced differentiation of blood MO.[20]

In this study, the effect of 1,25$(OH)_2D_3$ on the induction and promotion of terminal human MO to MAC differentiation in serum-free culture was investigated. MO were found to develop all the characteristics of mature MAC with 1,25$(OH)_2D_3$ being the only additive to the culture medium. Successful terminal differentiation was shown not only by the expression of maturation-associated (MAX) antigens but also by the cytokine repertoire of 1,25$(OH)_2D_3$-induced MAC.

MATERIALS AND METHODS

MO culture. Peripheral blood mononuclear cells (MNC) were separated from leukapheresis products of healthy donors by density gradient centrifugation over Ficoll. MO were isolated from MNC by countercurrent centrifugal elutriation in a J6M-E centrifuge and a JE-5 rotor (Beckman, München, Germany) at 2,500 rpm with a standard chamber and a flow rate of 20 mL/min as previously described.[10] Elutriated MO were of greater than 90% purity as estimated by morphology and expression of CD14 antigen. Purified MO were resuspended in RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 5×10^{-8} mol/L mercaptoethanol, vitamins, antibiotics, pyruvat, and nonessential amino acids. MO were cultured in 96-well microtiterplates (Greiner, Nürtingen, Germany) at 2×10^{-5} cells/0.2 mL supplemented RPMI 1640 with or without 5% AB-group serum or 1,25$(OH)_2D_3$ (Hoffmann-La Roche, Basel, Switzerland) at various concentrations. 1,25$(OH)_2D_3$ was dissolved in 100% ethanol to a stock concentration of 2×10^{-3} mol/L and stored at −20°C. The various concentrations were obtained by diluting the stock solution in RPMI 1640. After 7 days medium was removed and cells were cultured with fresh, serum-free supplemented RPMI 1640 without or with 1 μg/mL lipopolysaccharide (LPS) (*Salmonella abortus equi*, kindly provided by C. Galanos, Max-Planck-Inst., Freiburg, Germany) for 24 hours. Supernatants were harvested and stored at −20°C.

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Immunophenotype analysis. Subsequent to supernatant collection cell monolayers were fixed with 0.05% glutaraldehyde and surface antigen expression was measured by cell enzyme-linked immunosorbent assay (ELISA) as described elsewhere.[9,10] The following antibodies were used: anti-β2-microglobulin (Becton Dickinson, Rödermark, Germany), MAX.1 and MAX.3 (own laboratory).[8] Optical density (OD) was measured at 492 nm, corrected by the OD of specificity controls, mean of triplicates. Data are given as antigen expression index (AEI), which was calculated by dividing the OD values of the respective antigen by the OD value for β2-microglobulin expression times 100.

Functional analysis. Supernatants were pooled from 15 individual wells and tested for tumor necrosis factor α (TNFα) by ELISA (T Cell Science, Inc, Cambridge, MA). Fibronectin was also determined by ELISA (own development). Lysozyme was measured photometrically (Behringwerke, Marburg, Germany). Interleukin-6 (IL-6) activity was tested in a bioassay using the IL-6–dependent cell line B9 (kindly provided by Dr Aarden, Amsterdam, The Netherlands).[21]

RESULTS

The in vitro differentiation of circulating blood MO to MAC occurs in the presence of serum and can be followed by the expression of maturation-associated antigens of the MAX series that are absent on blood MO but expressed on terminally differentiated MAC. As shown in Fig 1, $1,25(OH)_2D_3$ can substitute for serum to promote the differentiation of MO into MAC as determined by the expression of MAX.1 and MAX.3 antigens. In the range of 1 to 100 nmol/L $1,25(OH)_2D_3$ MAX antigen expression was comparable with that of serum-derived MAC. There was no maturation in the absence of serum or $1,25(OH)_2D_3$. This result was also evident from morphology: in serum-free cultures cells stayed monocytoid in morphology, whereas MO treated with serum or $1,25(OH)_2D_3$ became larger, more spread, and developed macrophage-typical morphology (not shown).

These phenotypic and morphologic changes taking place during the maturation from MO into MAC are accompanied by characteristic changes in the biosecretory repertoire of the cell. MO were cultured for 7 days with or without serum or $1,25(OH)_2D_3$ and day-7 supernatants analyzed for the following secretory products: lysozyme, fibronectin, IL-6, and TNFα.

As shown in Fig 2, $1,25(OH)_2D_3$-induced MAC produce large amounts of IL-6 when stimulated with LPS. The secretion is about sixfold higher than serum-derived MAC. TNFα, which increases 10-fold on MO to MAC maturation, is in the same range in serum and $1,25(OH)_2D_3$-induced MAC (Fig 2). Lysozyme and fibronectin are secreted constitutively in similar amounts by $1,25(OH)_2D_3$- and serum-induced MAC, respectively (Fig 3). The optimal dose of $1,25(OH)_2D_3$ to induce functionally competent MAC in terms of cytokine release was 1 to 100 nmol/L $1,25(OH)_2D_3$, which is similar to what was observed for the expression of maturation-associated antigen.

Thus, from phenotypic and functional analysis $1,25(OH)_2D_3$-induced MAC resemble mature serum-derived MAC.

DISCUSSION

The in vitro maturation of blood MO into MAC is a useful model to study regulatory signals and functional consequences of this differentiation process, which seems to be central in the ontogeny of the MAC cell system.[1] Factors present in serum and responsible for this differentiation-inducing activity have not been identified yet. There have been reports implicating M-CSF,[11] IL-4,[22] and human gamma globulins[23] as possible mediators for MAC differentiation. However, none of these substances had the capacity to induce MAC differentiation in our culture system comparable with serum though M-CSF proved to be essential for MO survival in vitro.

Our results presented here clearly show that the active metabolite of vitamin D3, $1,25(OH)_2D_3$, induces terminal differentiation of circulating blood MO to mature MAC in vitro. Macrophages developing in the presence of $1,25(OH)_2D_3$ expressed maturation-associated antigens and secreted lysozyme and fibronectin with the latter being restricted specifically to MAC at differentiation stages beyond the blood MO level[24,25] In addition, the functional competence of $1,25(OH)_2D_3$-induced MAC is shown by their

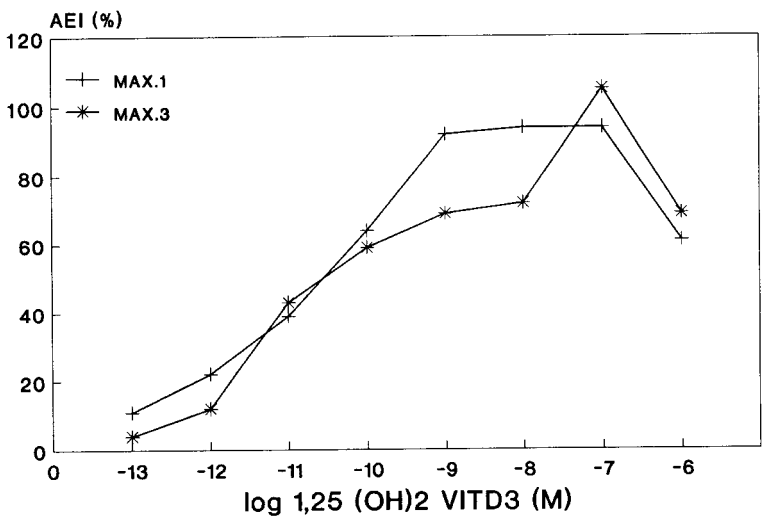

Fig 1. Induction of MO to MAC maturation by $1,25(OH)_2D_3$. Elutriated MO were cultured with $1,25(OH)_2D_3$ for 7 days in microtiterplates. Maturation is analyzed by the expression of MAX.1 and MAX.3 antigens given as AEI, mean of triplicate values, SD <15%. Data are from one representative experiment out of three.

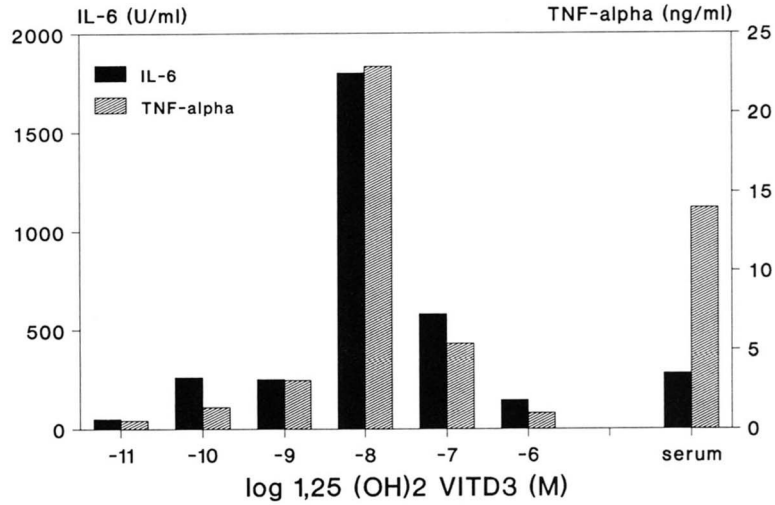

Fig 2. LPS-induced secretion of IL-6 and TNFα in $1,25(OH)_2D_3$-induced MAC. MO were cultured for 7 days with serum or $1,25(OH)_2D_3$, respectively. Medium was removed and MAC were cultured for 24 hours with fresh medium containing 1 μg/mL LPS abortus equi. Supernatants were then tested for cytokines.

cytokine repertoire, eg, high release of TNFα and IL-6 and the absence of IL-1β (not shown) a feature common to successful MO to MAC differentiation in vitro.[26,27] Although $1,25(OH)_2D_3$ has been long known as an inducer of monocytic differentiation[15-20] and osteoclast generation,[28] most published work was concerned with the differentiation of tumor cell lines and early hematopoietic precursor cells. There are some lines of evidence that MO to MAC differentiation is differently regulated than monocytic differentiation of tumor cell lines. First, the differentiation induced in tumor cell lines seems not to proceed beyond the blood MO stage (unpublished observation). Secondly, the differentiation-inducing stimulant for tumor cells, interferon-γ (IFNγ), has opposite effects on blood MO, eg, suppresses serum-induced maturation to MAC.[10] In comparison with serum-containing cultures $1,25(OH)_2D_3$-induced MAC maturation resulted in a considerable smaller cell recovery (20% to 40% of serum-induced MAC). Additional factors may be present in serum to promote cell survival. Studies are in progress to identify those cofactors that might be present in the albumin fraction as shown by preliminary experiments.

It should be noted that the effects observed in our system are in a dose range about 10- to 100-fold higher than measured in the AB-group sera used in our experiments (mean of six different donors 1.1×10^{-10} mol/L, SD = 5×10^{-11} mol/L; measured by Limbach, Schmidt-Gayk, Heidelberg, Germany). This level of $1,25(OH)_2D_3$ is comparable with serum levels reported by other groups (1×10^{-10} mol/L).[29] Yet it should be noted that this is an effect induced by $1,25(OH)_2D_3$ alone in serum-free culture, whereas in serum other synergistic factors have to be discussed that may potentiate the effect of $1,25(OH)_2D_3$. It should also be mentioned that for all in vitro activities attributed to $1,25(OH)_2D_3$, a similar dose is reported.[17-19] The negative effect of high doses of $1,25(OH)_2D_3$ most likely relates to an unspecific membrane effect because secosteroids like $1,25(OH)_2D_3$ are highly lipophilic substances. According to this finding is the observation that high doses of the metabolite $25(OH)D_3$ were found to be toxic for MO, too (data not shown in detail).

Elevated serum levels of $1,25(OH)_2D_3$ have been shown in diseases such as sarcoidosis and macrophages associated with granuloma tissue secrete $1,25(OH)_2D_3$ in vitro.[30] It is conceivable that MAC-derived $1,25(OH)_2D_3$ may serve as an

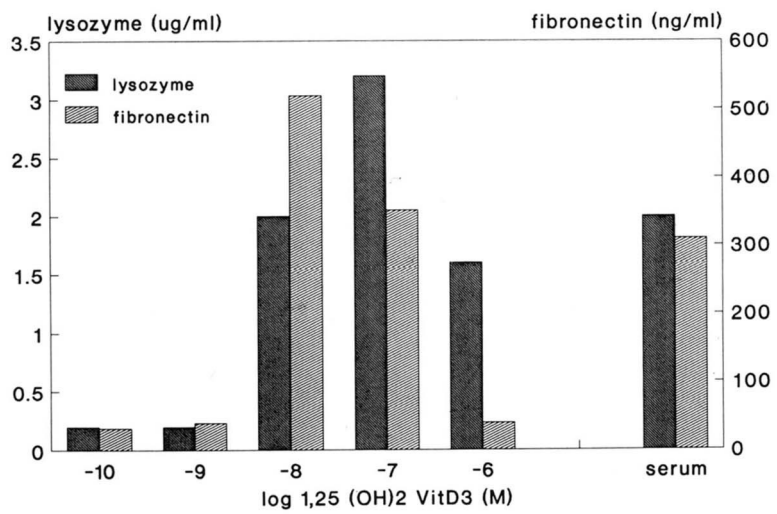

Fig 3. Constitutive secretion of lysozyme and fibronectin by $1,25(OH)_2D_3$-induced MAC. For details see legend to Fig 2. Supernatants were generated without the addition of stimuli.

autocrine signal to promote MO to MAC generation and may even be implicated in the pathophysiology of this chronic inflammatory reaction by inducing that type of MAC characteristic of a granulomatous inflammation. Chronic bacterial infection may support this autoregulatory circuit as LPS are potent stimuli of vitamin D3 metabolism.[31] Similarly, IFNγ stimulates the synthesis of $1,25(OH)_2D_3$ in normal human MAC.[32] It should also be noted that MAC obtained from vitamin D3-deficient mice showed an impaired response to activation for tumor cytotoxicity.[33] In view of our data this functional defect might well be related to incomplete MO maturation as both the spontaneous and IFNγ-activated tumor cytotoxicity depend crucially on a successful differentiation of blood MO to mature MAC.[34]

In conclusion, the active vitamin D3 metabolite $1,25(OH)_2D_3$ appears to participate in the regulation of MAC ontogeny not only at the level of committed stem cells but also as inducer of normal MO to MAC maturation. Especially the latter activity, through the multifold interactions of the MPS with other cell systems, may have widespread implications for normal homeostasis as well as to understand the pathophysiology of abnormal vitamin D3 metabolism as it is seen in granulomatous disorders, hematopoietic neoplasia, and chronic renal failure.

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