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Expression of Retinoid Receptors during Human Monocyte Differentiation *in Vitro*

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1α,25-Dihydroxyvitamin D_3 (1,25$(OH)_2VD_3$) and retinoic acid (RA) modulate the activation of monocytes (MO) and their differentiation into macrophages (MAC). As these effects are mostly mediated by heterodimers or homodimers of the specific nuclear receptors for 1,25$(OH)_2VD_3$ and RA, we investigated the expression of the retinoic acid receptors (RAR) α, β, and γ and the retinoid X-receptor (RXR) α in MO during differentiation into MAC or dendritic cells (DC). The mRNA of all investigated receptors except RARβ was detected in short-term cultured MO. During differentiation of MO to MAC the mRNA expression of the RA receptors decreased. In contrast, along the differentiation pathway of MO to DC, only the mRNA expression of RARγ declined, whereas RARα and RXRα were constantly expressed at a high level. Despite the strong expression of RARα and RXRα at mRNA level in MO-derived DC, the protein expression of the receptors was low in these cells. However, MO and MO-derived MAC showed a strong expression of these receptors at protein level. This suggests that a posttranscriptional or posttranslational mechanism of receptor regulation is occurring in these cells, and in particular in the DC. The inverse regulation of RA receptor expression and protein levels between MAC and DC may control the responsiveness of these cells to 1,25$(OH)_2VD_3$ and RA. © 2000 Academic Press

Human blood monocytes (MO) originating from the bone marrow are the common precursor cells for macrophages (MAC) and dendritic cells (DC) (1, 2). *In vitro* several differentiation models of MO are established. For example, MO differentiate along the MAC pathway by culture with human serum (3) and along the DC pathway by culture with fetal calf serum (FCS), IL-4 and GM-CSF (4–6).

The differentiation process of various cell types, including myelomonocytic cell lines and MO, is modulated by the active forms of vitamin D and vitamin A, 1,25$(OH)_2VD_3$ and retinoic acid (RA), acting either in synergistic or antagonistic fashion depending on the concentration and/or cell type. In the human myelomonocytic cell line HL-60, the induction of the differentiation into MO/MAC by 1,25$(OH)_2VD_3$ is supported by low RA concentrations (7), while high RA concentrations alone induce the differentiation of HL-60 cells into neutrophils (8). With regard to the *in vitro* differentiation of primary human blood MO to MAC, 1,25$(OH)_2VD_3$ affects this differentiation process positively by upregulation of carboxypeptidase M/MAX.1 expression (9) and M-CSF production (10). In contrast, RA downregulates carboxypeptidase M/MAX.1 expression and reduces the survival rate of MO by suppression of M-CSF production (11). Concerning differentiation of MO to DC, an inhibitory effect of 1,25$(OH)_2VD_3$ has been shown (12), but for retinoids both positive and negative effects have been described (13, 14).

1,25$(OH)_2VD_3$ and RA mediate their genomic effects by specific nuclear receptors belonging to the superfamily of nuclear steroid-, thyroid- and retinoid-receptors. For 1,25$(OH)_2VD_3$ only one receptor, the vitamin D receptor (VDR), has been described (15), but for RA two different types of receptors are known, each with at least 3 isoforms (α, β and γ). These are the retinoic acid receptor (RAR) (16–19) and the retinoid-X-receptor (20–22). The retinoid receptors differ in their ligand specificity: RAR binds all-*trans* and 9-*cis* RA (23), but RXR binds only 9-*cis* RA (24). Most members of this superfamily interact with each other and form homodimers and/or heterodimers, which bind to specific response elements of target genes and affect their transcription (25). For the 1,25$(OH)_2VD_3$ re-

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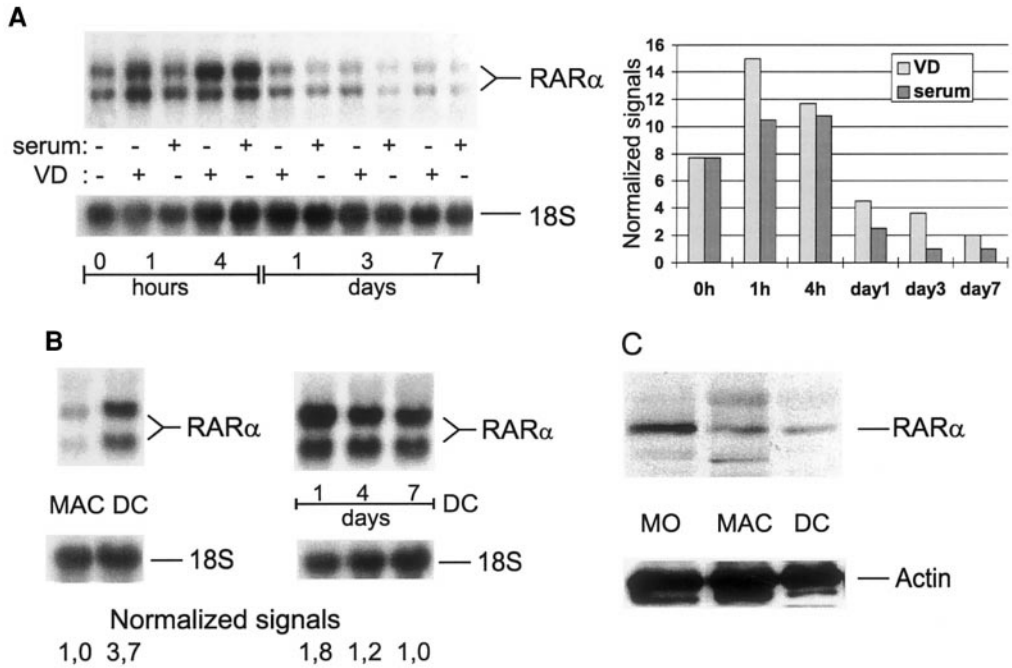

FIG. 1. Expression of RARα during MO to MAC (A) or DC differentiation (B) *in vitro.* Human blood MO were cultured with 2% serum and under serum-free conditions with 10^{-7}M $1,25(OH)_2VD_3$ (for MAC differentiation) or with FCS, IL-4, and GM-CSF (for DC differentiation) for up to 7 days. The expression of RARα mRNA was analyzed by Northern blot analysis and values of RARα expression were normalized to the respective 18S signals. RARα protein was measured by Western blot analysis in MO, MAC, and DC. The content of the used protein lysates were controlled by staining with an anti-actin antibody (C).

sponse mainly heterodimers of RXR (and RAR) with VDR (26–28) are decisive, for the RA response heterodimers of RXR with RAR or homodimers of RXR (29–32).

The VDR and retinoid receptors are ubiquitously expressed. In primary myeloid cells, VDR expression is found in MO, MO-derived MAC (33, 34) and dendritic cells from tonsils (35), but up to now no data have been available concerning the expression and regulation of RAR and RXR during differentiation of human blood MO into MAC or DC. As the retinoid receptors are important for both RA and $1,25(OH)_2VD_3$ responses, in this study we have investigated the repertoire of these receptors during the differentiation of MO to MAC or DC *in vitro.*

MATERIALS AND METHODS

Chemicals. All chemical reagents used were purchased from Sigma (Deisenhofen, Germany) unless otherwise noted. $1,25(OH)_2VD_3$ was kindly provided by Hoffmann-LaRoche, Basel, Switzerland and human recombinant IL-4 by Schering-Plough, New Jersey. The anti-RARα (clone: D-20) and anti-RXRα (clone: C-20) antibodies were purchased from Santa Cruz (Santa Cruz, CA). The full-length cDNA of RXRα (22) was kindly provided by L. Freedman (New York, NY) and the full-length cDNAs of RARα (19), RARβ (18, 36) and RARγ (17) by A. Boβerhoff (Regensburg, Germany).

Cell separation and culture. Peripheral human blood mononuclear cells were isolated by leukapheresis of healthy donors. Following the gradient centrifugation over Ficoll/Hypaque (Pharmacia, Freiburg, Germany) purified MO were obtained by countercurrent centrifugation in a J6M-E centrifuge (Beckmann, München, Germany), as described previously (37). The purity (>95%) of the MO was determined by morphology and expression of CD14 antigen. MO were cultured at a density of 10^6 cells/ml with RPMI (Biochrom, Berlin, Germany) supplemented with 50 mM mercaptoethanol, antibiotics (0.5 U/ml penicillin and 0.5 mg/ml streptomycin), 1 mM pyruvate, 1× MEM non-essential amino acids (Gibco BRL, Eggenstein, Germany), 1× MEM vitamins (Gibco BRL), 0.22 mg/ml L-glutamine (Gibco BRL).

MAC were generated in petri dishes by culturing MO for up to 7 days with 2% AB-serum (Sigma, Deisenhofen, Germany) or under serum-free conditions with 10^{-7}M $1,25(OH)_2VD_3$. DC were obtained by cultivation of MO in culture flasks for up to 7 days with 10% FCS (c.c.pro, Karlsruhe, Germany), 35 ng/ml human recombinant GM-CSF (Sandoz-Essex, Munich, Germany) and 500 U/ml human recombinant IL-4 (Schering-Plough, New Jersey).

RNA preparation. Total RNA was isolated from primary cells by the method of Chomczynski and Sacchi (38).

Northern blot analysis. Total RNA (10 μg/lane) was electrophoretically separated on an 1% agarose formaldehyde gel, transferred to nylon membranes (Magna NT, MSI, Westborough, MA) and UV cross-linked. The cDNA of RARα and RARγ were phospholabelled with gene-specific primers and RXRα with hexamers by the "Random Primed Labelling Kit" (Boehringer, Mannheim, Germany). Hybridization was performed over night at 65°C in church buffer (0.5 M sodiumphosphate buffer pH 7.2, 7% SDS, 1 mM EDTA and 150 μg/ml tRNA) (39). In control experiments membranes were rehybridized with an 18S rRNA-oligonucleotide (5′-ACG GTA TCT GAT CGT CTT CGA ACC-3′) labelled by T4 Kinase ("5′-End Labelling Kit", Amersham, Buckinghamshire, UK). After autoradiography the signals were measured by densitometry and the specific values were normalized to the 18S values.

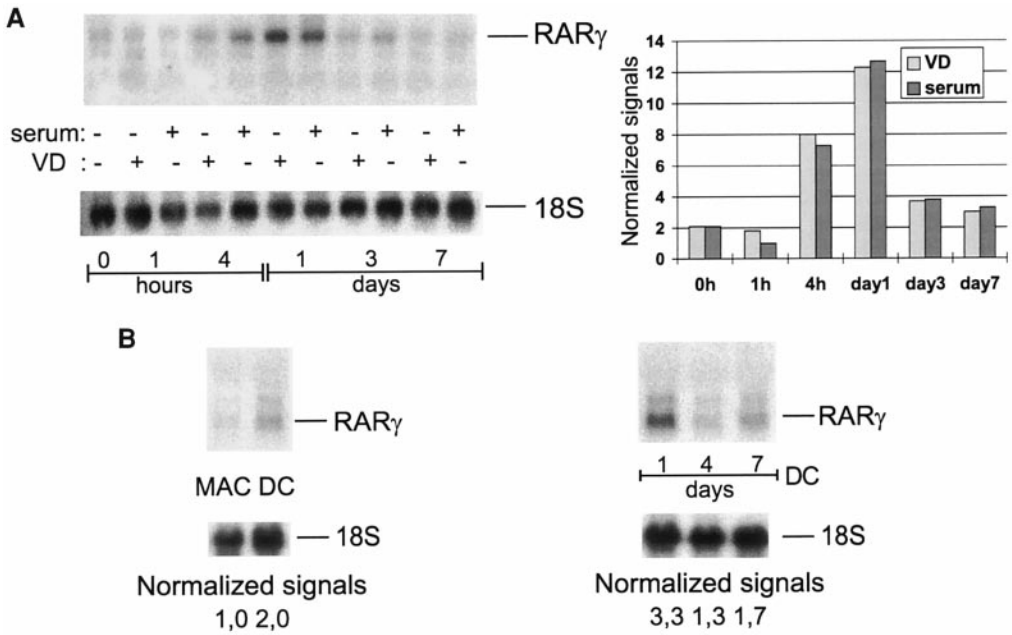

FIG. 2. Expression of RARγ during MO to MAC (A) or DC differentiation (B) *in vitro.* Human blood MO were cultured with serum and under serum-free conditions with $1,25(OH)_2VD_3$ (for MAC differentiation) or with FCS, IL-4, and GM-CSF (for DC differentiation) for up to 7 days. The expression of RARγ mRNA was analyzed by Northern blot analysis and values of RARγ expression were normalized to the respective 18S signals.

Western blot analysis. After lysis of the cells (60 mM Tris/HCl with pH 6.8, 2% SDS, 5 mM EDTA, 10 mM DTT, 1 mM PMSF) the samples were boiled for 10 min at 95°C, separated on 10% SDS-PAGE gels and transferred to Hybond-ECL membranes (Amersham, Birmingham, UK). Membranes were incubated for 2 h in blocking buffer (PBS with 3% BSA) before incubation for 1 h in blocking buffer containing the antibody in a concentration of 0.4 mg/ml and 0.05% Tween 20. After three washing steps with blocking buffer containing 0.05% Tween 20, membranes were incubated for 1 h in blocking buffer with horseradish peroxidase-conjugated swine anti-rabbit immunoglobulins (1/500 diluted; DAKO, Hamburg, Germany) and 0.05% Tween 20. After three washes in PBS with 0.05% Tween 20, signals were developed with the ECL Western blotting reagents (Amersham, Birmingham, UK) according to the manufacturer's instructions and detected by exposition to Kodak film.

RESULTS

RARα, β, and γ Expression during in Vitro Differentiation of MO into MAC and DC

The retinoid receptors mediate the RA effects and are also important for the $1,25(OH)_2VD_3$ response in the respective target cells. As myeloid cells are target cells for RA and $1,25(OH)_2VD_3$, we were interested in the expression pattern of the different RAR isoforms and RXRα in MO, MO-derived MAC and MO-derived DC.

In order to induce MAC differentiation we cultured MO serum-free with 10^{-7}M $1,25(OH)_2VD_3$ or with 2% human serum for up to 7 days (3, 40). In freshly isolated MO the expression of RARα mRNA was detected, which was increased after 4 h of culture and then slightly downregulated. In MAC only a weak RARα mRNA expression was found. A similar pattern of RARα mRNA expression was observed either with serum or under serum-free conditions with $1,25(OH)_2VD_3$ (Fig. 1A). In contrast, MO cultured along the DC pathway with 10% FCS, 500 U/ml IL-4 and 40 ng/ml GM-CSF (5, 41, 42) expressed RARα mRNA at a high level on day 1 of culture and maintained this strong expression during the whole differentiation process (Fig. 1B).

On the basis of the strong expression of RARα at mRNA level we were interested in the expression of RARα at protein level in MO, MO-derived MAC and DC respectively. Similarly to the mRNA findings, freshly isolated MO showed strong expression of RARα protein, and in MO-derived MAC after 7 day of culture RARα protein was only weakly expressed. However, in contrast to the RARα mRNA signal in MO-derived DC, only weak expression of RARα protein was found in these cells (Fig. 1C).

In addition, we also investigated expression of RARβ mRNA, but this was almost completely undetectable in MO, MAC and DC (data not shown) and we did not perform further Western blot analysis for this protein.

Compared with RARα, RARγ showed a totally different expression pattern during MO to MAC differentiation. In freshly isolated MO only weak signals of RARγ mRNA were detected, but the expression increased after one day of culture. After further cultivation this expression declined to the base level (Fig. 2A).

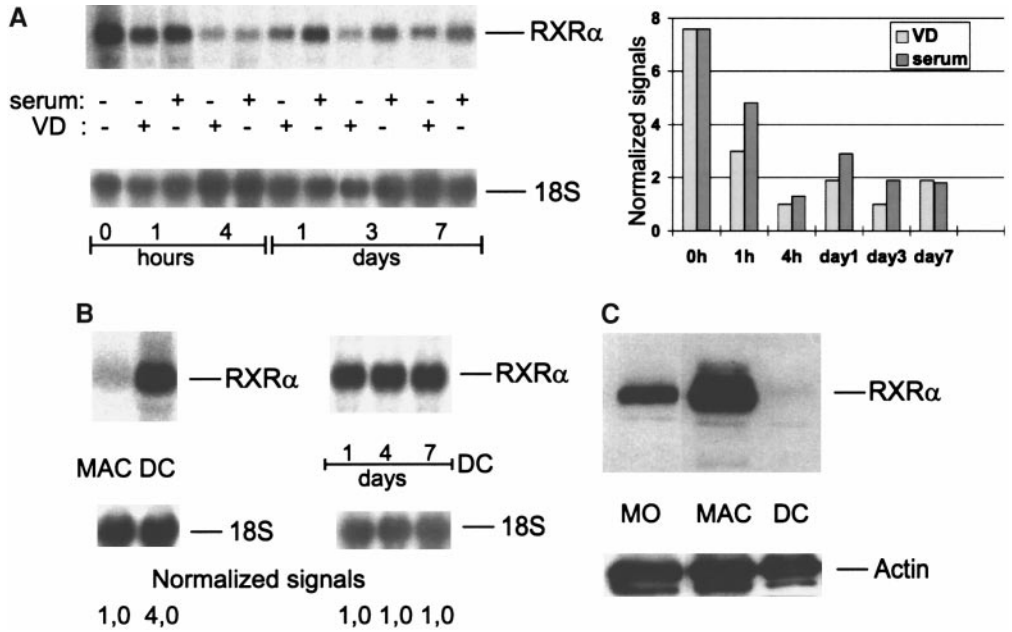

FIG. 3. Expression of RXRα during MO to MAC (A) or DC differentiation (B) *in vitro.* Human blood MO were cultured with serum and under serum-free conditions with $1,25(OH)_2VD_3$ (for MAC differentiation) or with FCS, IL-4, and GM-CSF (for DC differentiation) for up to 7 days. The expression of RXRα mRNA was analyzed by Northern blot analysis and values of RXRα expression were normalized to the respective 18S signals. RXRα protein was measured by Western blot analysis in MO, MAC, and DC. The content of the used protein lysates were controlled by staining with an anti-actin antibody (C).

Also during differentiation of MO into DC, RARγ mRNA expression was upregulated in short-term culture and decreased after seven days, i.e. directly comparable to the expression findings in MO-derived MAC (Fig. 2B).

RXRα Expression during in Vitro Differentiation of MO into MAC and DC

The most important putative dimerization partner of both VDR and RAR is RXR, which can also mediate the RA response independent of RAR as a homodimer. Therefore this retinoid receptor may play a key role in effects induced by both RA and $1,25(OH)_2VD_3$. As most interaction studies between other receptors and RXR have been performed with RXRα, we investigated the expression of this particular isoform in our system.

In freshly isolated MO we detected a strong signal of RXRα mRNA expression. During *in vitro* differentiation of MO to MAC this strong signal was downregulated, but was still found in MAC generated under both serum-free and serum-containing conditions (Fig. 3A). In MO cultured along the DC pathway the strong RXRα expression was maintained and MO-derived DC showed strong expression of RXRα mRNA, similar to that seen in freshly isolated MO (Fig. 3B). Surprisingly, however, the results of the Western blot analysis were not in line with the data obtained in the Northern blots. At protein level freshly isolated MO expressed significant amounts of RXRα protein and during differentiation of MO to MAC the expression was strongly upregulated, whereas during differentiation of MO to DC the RXRα protein expression was downregulated completely (Fig. 3C).

To summarize these findings, Table I shows the comparative expression of the RARα and RXRα mRNA and protein findings in freshly isolated MO, MO-derived MAC and MO-derived DC.

DISCUSSION

It is generally accepted that cells belonging to the monocyte lineage are targets for both RA and $1,25(OH)VD_3$, but the expression of the retinoid recep-

TABLE I

Expression of RARα and RXRα at mRNA and Protein Level during the *in Vitro* Differentiation of MO into MAC or DC

	MO day 0		MAC day 7		DC day 7	
	mRNA	Protein	mRNA	Protein	mRNA	Protein
RARα	++	++	+	+	+++	+
RXRα	+++	++	+	+++	+++	−

Note. The relative intensity of the respective expression is shown (+++: very strong expression, ++: strong expression, +: weak expression, +/−: very weak expression, −: no expression).

tors in primary human MO has not been investigated previously. In this study, therefore, we have analyzed the expression of the RA receptors, RARα, β and γ and RXRα, during *in vitro* differentiation of MO into MAC or DC.

The results obtained by Northern blot analysis showed correlated expression of RARα protein both in freshly isolated MO and in MO-derived MAC. In contrast, for RXRα there was an inverse relationship between protein and mRNA levels. Further discrepancies between protein and mRNA expression were found in MO-derived DC, where there was only weak expression of both RARα and RXRα proteins despite the presence of abundant mRNA for these receptors. This implies that for RXRα there is probably a posttranscriptional or posttranscriptional mechanism of receptor regulation which operates in both MAC and DC; and that in DC this also applies to RARα. This resembles the differences between expression of mRNA and protein that have also been observed for other members of the steroid-, thyroid- and retinoid receptor superfamily, e.g., peroxisome proliferation-activated receptors, corticosteroid receptors and estrogen receptor, and raises the possibility of a similar mechanism of regulation (43–45). One possible mechanism of receptor regulation could be a proteasome-dependent degradation similar to that described recently for RARα (46).

The regulation of several genes by RA or $1,25(OH)_2VD_3$ in freshly isolated MO, which has been shown by several investigators (47–50) correlates with the expression of the different retinoid receptors in these cells. During differentiation of MO into MAC the pattern of protein expression of the receptors changed: MO and MO-derived MAC expressed the RARα and VDR (data not shown) at a lower level, but the RXRα expression was significantly upregulated. This may be indicative of a shift of receptor dimerization pattern from VDR-RXRα-heterodimers to RXRα-RXRα-homodimers during the MAC differentiation pathway. As the $1,25(OH)VD_3$ synthesis is strongly increased during MAC differentiation (34), we suggest that the cells may protect themselves against autocrine $1,25(OH)VD_3$ stimulation and loose their sensitivity for $1,25(OH)_2VD_3$ by this shift to RXRα-RXRα-homodimers. This is consistent with the observations about RXRα expression in cell lines that have been reported previously (51) but there has been little analysis of effect of RA on MAC in this context, and the role of MAC themselves as a site of RA production is still unclear. It may be relevant that, in addition to the strong expression of RXRα, the expression of CRABPII mRNA, one of the two cellular retinoic acid binding proteins, also increases during differention of MO into MAC (52). This may be important for the RA response, because CRABP II is involved in the catabolism of RA (53, 54) and/or in the transport of RA into the nucleus (55). The hypothesis that there is a synergistic interaction between RXRα and CRABP II is supported by the observation that downregulation of CRABP II inhibited RA response (56).

In contrast to MO-derived MAC, MO-derived DC completely downregulated the expression of RXRα at protein level, probably via a posttranscriptional or posttranslational mechanism and only weakly expressed RARα protein. As CRABP II mRNA is also decreased during the *in vitro* differentiation of MO into DC (47), we suggest that there is only a minor response of DC to RA. In mice, where most of the studies have been performed, the effects of RA on cells of this lineage remain controversial. *In vitro* retinoids have been shown to have a negative influence on the accessory function of DC in a mixed lymphocyte reaction (13), but if given *in vivo* retinoids had no direct effect on functional properties, and any change in responsiveness pattern appeared to be due to an increased number of accessory cells (14). It is interesting that this bears some correlation to a human system where an increase in the number of Langerhans cells after application of tretionin to the skin has been observed (57). The increase in the number of DC in both the murine and human systems may be indicative of a positive effect of RA on DC precursor cells, e.g., MO, which express the respective receptors, and not on mature DC, which lack the receptors. Thus further investigation to clarify both the control of retinoid receptor expression and its effects on DC function may help to explain how differential function is achieved in this lineage.

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