Journal of Cellular Biochemistry 88:986–998 (2003)

Modulation of TNF-α Expression in Bone Marrow Macrophages: Involvement of Vitamin D Response Element

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Abstract The calcium-regulating hormone, $1,25(OH)_2D_3$, induces tumor necrosis factor-α (TNF-α) synthesis and release from bone marrow macrophages (BMMs). To investigate the mechanism of this regulation, we have examined the effects of $1,25(OH)_2D_3$ on the cytokine message. $1,25(OH)_2D_3$ increased TNF-α mRNA abundance in a dose- and time-dependent manner. The combined treatment of BMMs with LPS and $1,25(OH)_2D_3$ resulted in a synergistic increase of TNF-α. The steroid also increased the expression of CD14 (LPS receptor). Vitamin D receptors (VDRs) mediate $1,25(OH)_2D_3$ genomic effects by forming homodimers or heterodimers with retinoic acid receptors (RARs) or retinoic X receptors (RXRs). The RXR ligand, 9-*cis* retinoic acid (9cRA), reduced TNF-α mRNA abundance in BMMs, but increased CD14 mRNA levels. $1,25(OH)_2D_3$ or LPS did not affect TNF-α transcript stability. 9cRA, however, caused TNF-α mRNA destabilization. Next, we searched for potential vitamin D response elements (VDREs) in the promoter region (1.2 kb) of the TNF-α gene, and identified six such sequences. Using electrophoresis mobility shift assay (EMSA) we identified one of those sequences (−1008 to −994) as a likely candidate to be a VDRE (tnfVDRE). The binding of tnfVDRE to BMM-derived nuclear extract was increased following cell treatment with $1,25(OH)_2D_3$. No induction was observed with 9cRA treatment, but the retinoid enhanced the activity of $1,25(OH)_2D_3$ when added together. Previously characterized VDREs (mouse osteopontin and rat osteocalcin) competed effectively with tnfVDRE, demonstrating the nature of the TNF-α-derived sequence as a VDRE. We observed super-shift and block-shift of the complex in the presence of either anti-VDR or anti-RXR antibodies. Our data suggest that $1,25(OH)_2D_3$ increases TNF-α transcript abundance in BMMs via a transcriptional mechanism; 9cRA decreases TNF-α mRNA by destabilizing the transcript, and possibly also by forming transcriptionally inactive complex with $1,25(OH)_2D_3$ on the tnfVDRE. The receptor complex interacting with tnfVDRE found in the promoter of the cytokine gene is probably composed of VDR-RXR heterodimer. J. Cell. Biochem. 88: 986–998, 2003.

Key words: $1,25(OH)_2D_3$; retinoic-acid; VDR; RXR; lipopolysaccharide; CD14

The active metabolite of vitamin D, 1,25-dihydroxyviatmin D_3 ($1,25(OH)_2D_3$), in addition to its classical role in calcium homeostasis, regulates the growth, differentiation, and function of a broad range of cells, including cells of the immune system [Provvedini et al., 1983; DeLuca, 1988; Kumar et al., 1989; Pike, 1991; DeLuca and Cantorna, 2001]. Studies in the last two decades assigned immunomodulatory roles to $1,25(OH)_2D_3$, and cells belonging to the immune system contain functional vitamin D receptors (VDRs) [Manolagas et al., 1985; Bhalla et al., 1986; Minghetti and Norman, 1988; Rigby, 1988].

Macrophages derived from vitamin D-deficient mice exhibit impaired functions [Bar-Shavit et al., 1981; Gavison and Bar-Shavit, 1989; Kankova et al., 1991; Abu-Amer and Bar-Shavit, 1993]. $1,25(OH)_2D_3$ has been shown to induce in vitro and in vivo monocyte/macrophage differentiation and activation in cell lines as well as in primary cells [Abe et al., 1981; Bar-Shavit et al., 1983; Amento et al., 1984; Tokuda et al., 1992; Abu-Amer and Bar-Shavit, 1993; Panichi et al., 1998; Cohen et al., 2001].

Grant sponsor: ICRF.

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Received 28 October 2002; Accepted 29 October 2002
DOI 10.1002/jcb.10453

Mononuclear phagocytes play a pivotal role in initiating and maintaining the immune response and produce numerous biologically active molecules, including cytokines such as tumor necrosis factor-α (TNF-α) [Old, 1985; Fiers, 1991; Beutler, 1999]. TNF-α expression is regulated by transcriptional and post-transcriptional mechanisms [Han and Beutler, 1990; Han et al., 1990; Biragyn and Nedospasov, 1995; Crawford et al., 1997; Raabe et al., 1998; Anderson, 2000]. The macrophage activating molecule, lipopolysaccharide (LPS) is a major inducer of TNF-α synthesis and release [Han et al., 1990; Prehrn et al., 1992; Rietschel and Brade, 1992; Kilbourn et al., 1993; Fenton and Golenbock, 1998; Giovannini et al., 2001]. LPS interacts with membrane receptors (CD14) on the target cells [Han et al., 1990; Rietschel and Brade, 1992; Kilbourn et al., 1993; Biragyn and Nedospasov, 1995; Delude et al., 1995; Fenton and Golenbock, 1998]. CD14 binds LPS and subsequently initiates cellular activation [Wright et al., 1990; Ulevitch and Tobias, 1995]. CD14 transduces its signals via interactions with the toll-like receptors (TLRs) [Delude et al., 1995; Kirschning et al., 1998; Yang et al., 1998; Kopp et al., 1999; Takashi et al., 2000; Tapping et al., 2000; Takeuchi and Akira, 2001; Tsan et al., 2001].

Genomic effects of $1,25(OH)_2D_3$ are mediated by VDR that activates transcription by binding to vitamin D response elements (VDREs) within the promoters of vitamin D responsive genes [Evans, 1988; Haussler et al., 1988; O'Malley, 1990; Norman et al., 1992; Wu et al., 1999], either as a homodimer [Forman et al., 1992; Carlberg et al., 1993], or as a heterodimer with the retinoic acid X receptor (RXR) [Yu et al., 1991; Kliewer et al., 1992; Leid et al., 1992; Carlberg, 1993; Staal et al., 1996; Tsonis et al., 1996; Zou et al., 1997; Prufer and Barsony, 2002]. VDREs are comprised of direct repeats (DRs), palindromes (Ps), or inverted palindromes (IPs) of the hexameric core binding motif RRKNSA (R = A or G, K = G or T, S = C or G) [Carlberg, 1993; Schrader et al., 1994, 1995].

We have previously demonstrated that $1,25(OH)_2D_3$ increases the TNF-α synthesis, and addition of LPS further increase the synthesis and release of this cytokine [Abu-Amer and Bar-Shavit, 1994]. In the present study we examine the modulation of TNF-α mRNA by $1,25(OH)_2D_3$.

MATERIALS AND METHODS

Reagents

1,25-Dihydoxyvitamin D_3 ($1,25(OH)_2D_3$) was purchased from Biomol (Plymouth, PA). 9-*cis*-Retinoic acid (9cRA) was purchased from Sigma (St. Louis, MO). Lipopolysacharide (LPS, *Escherichia coli* serotype 0111:B4) was purchased from Difco laboratories (Detroit, MI). Minimum essential medium alpha (α-MEM) and charcoal stripped fetal calf serum (CH-FCS) were purchased from Biological Industries (Beit-Haemek, Israel). Rat monoclonal anti-mouse-CD14 antibody (rm C5-3) was purchased from Pharmingen (San Diego, CA). Mouse monoclonal anti-VDR antibodies and anti-RXR antibodies were kindly provided by Dr. Lian [Staal et al., 1996]. Rabbit polyclonal anti-mouse RXR (mapped to amino acids 198–462) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Peroxidase-conjugated anti-rat-IgG and rabbit anti-mouse whole serum were purchased from Jackson Immunoresearch, Inc. (West Grove, PA). ECl kit was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were of analytical grade, and obtained from Sigma.

Mice

Seven to 9-week-old male BALB/c mice were obtained from Harlan laboratories Ltd. (Jerusalem, Israel). Vitamin D deficiency was obtained as described [Bar-Shavit et al., 1981].

Cells

Bone marrow derived macrophages (BMMs) were collected as described previously [Abu-Amer and Bar-Shavit, 1993]. Cells were collected from tibia and femurs and seeded into tissue culture plates in α-MEM containing 10% CH-FCS and 15% L-cell conditioned medium (as a source for macrophage colony stimulating factor), at 37°C in a humidified 5% CO_2 atmosphere. After 24 h, non-adherent cells were layered on a Ficoll-Hypaque gradient, and centrifuged in Sorvall-RT6000 (2000 rpm, 20 min). The interphase cells were removed and plated (10^7/10 ml, 100 mm tissue-culture plates).

Northern Blotting

Total RNA was extracted using the TRI-REAGENT kit (Molecular Research Center, Inc.) [Chomczynski, 1993]. The quality of the

RNA was verified by UV visualization of the ribosomal RNA bands (28S, 18S) of ethidium bromide stained agarose gel. RNA (10 μg/lane) electrophoresed on a 1.2% agarose-formaldhyde gel, and transferred overnight to Hybond-*N* nylon membrane (Amersham, UK). Then RNA membranes were hybridized with [^{32}P]-labeled TNF-α cDNA (1.1 Kb of murine TNF-α), [^{32}P]-labeled CD14 cDNA (477 bp of murine CD14), or the control housekeeping gene, [^{32}P]-labeled L32 (1 kb of L32). After hybridization, the membranes were washed and exposed to X-ray film (Fuji) at −80°C for autoradiography and the density of each mRNA band was quantified using Fluor-STM multiimager (Bio-Rad laboratories, Hercules, CA).

Western Blotting

Proteins were isolated using the TRI-REAGENT kit [Chomczynski, 1993]. Proteins (10 μg/lane) were electrophoresed on 12% SDS–PAGE, transferred to nitrocellulose membrane, and were incubated in blocking solution, to reduce non-specific binding. Membranes were incubated with the rat anti-mouse CD14 (overnight at 25°C), and then with Peroxidase-conjugated anti-rat-IgG antibody (4 h, at 25°C). An ECL detection assay was performed according to the manufacturer's instructions.

Electrophoresic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared [Staal et al., 1996]. [^{32}P]-double-stranded oligonucleotides containing the VDRE consensus sequence were used as probes (Table I). Nuclear extracts (2 μg) were incubated for 20 min at room temperature with the labeled probe and poly (dI-dC). Competition was carried out using 10–100 fold excess of the unlabeled oligonucleotides 15 min prior to probe addition. To identify the components of the VDR-complex, anti-VDR or anti-RXR antibodies were added for 45 min at room temperature. Samples were then subjected to electrophoresis in a 5% non-denaturing PAGE.

Statistical Analysis

Values were expressed as mean ± SD of n = 3. Student's *t*-test was used to determine of significance of differences.

RESULTS

We have previously shown that $1,25(OH)_2D_3$ induces TNF-α synthesis and release in BMMs [Abu-Amer and Bar-Shavit, 1994]. Here we show (Fig. 1) by Northern analysis that the steroid increases the abundance of TNF-α mRNA in these cells. The level of the cytokine mRNA in BMMs increases with time in culture. No significant change is observed from 24 to 48 h, but a marked increase (~5-fold) occurs between 48 and 72 h of incubation. The addition of $1,25(OH)_2D_3$ augments this increase. No significant effect of the vitamin D metabolite is observed in the first 2 days. After 72 h, a twofold increase in the steady state level of the cytokine mRNA is observed in BMMs treated with $1,25(OH)_2D_3$ (50 nM) as compared with BMMs grown in the absence of the hormone.

Both, $1,25(OH)_2D_3$ and LPS enhance the synthesis of TNF-α. Moreover, the release of TNF-α is induced by $1,25(OH)_2D_3$ only in the presence of LPS [Abu-Amer and Bar-Shavit, 1994]. We decided, therefore, to analyze the combined effect of $1,25(OH)_2D_3$ and LPS on the cytokine mRNA abundance. First, we confirm that LPS increases transcript abundance in a dose- and time-dependent manner (Fig. 2A,B, respectively). BMMs were incubated for 72 h in culture medium, and then challenged with LPS (time

TABLE I. Sequence of Established and Putative VDREs[a]

VDRE	5′-Half element	Spacer	3′-Half element
Mouse osteopontin	GGTTCA	CGA	GGTTCA
Rat osteocalcin	GGGTGA	ATG	AGGACA
Rat SHEmt	GtaTGA	ATG	ActACA
TNF-α (−690)	GAGTGA	A	AGGAGA
TNF-α (−1099)	AGTGGA	GC	AGGGGA
TNF-α (−1008)	GATTCA	CGG	GAGTGA
TNF-α (−1099)	AGTGGA	GCAG	GGGACA
TNF-α (−1019)	GAGACA	TGGTG	GATTCA
TNF-α (−952)	GATCCA	TCCAAG	GGTGGA

[a]The sequences were taken from Breen et al. [1994], Markose et al. [1990], Staal et al. [1996], and Iraqi and Teale [1997].

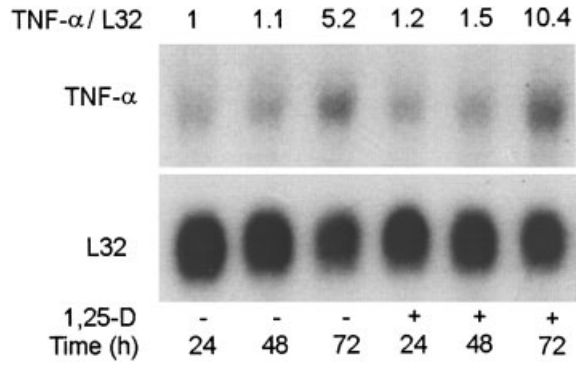

Fig. 1. Modulation of TNF-α mRNA abundance in BMMs by $1,25(OH)_2D_3$: Kinetics. Cells were incubated in the presence or absence of $1,25(OH)_2D_3$ (50 nM) for the indicated time. Then, total RNA was isolated, Northern-blotted, and analyzed for TNF-α and L32 (as a control gene) mRNA.

and dose as indicated). In Figure 2A, we see that already at 1 ng/ml of LPS (2-h incubation) a significant increase (40%) in the cytokine message abundance is observed. Twofold increase is obtained with 5 ng/ml of LPS. Figure 2B shows the kinetics of the LPS (5 ng/ml) induced increase in TNF-α mRNA abundance. The combined effect of $1,25(OH)_2D_3$ and LPS on TNF-α message abundance is shown in Figure 2C. BMMs were incubated for 72 h with $1,25(OH)_2D_3$ at different concentrations (0–50 nM), and then challenged with 5 ng/ml of LPS for 2 h. Control cultures were incubated for the last 2 h in the absence of LPS. As shown in Figure 2C, LPS augments the effect of the steroid. The hormone at 50 nM increased the mRNA levels by 190%, LPS alone by 90%, and the combination caused an increase of 490%, suggesting a positive synergistic effect.

This result prompted us to hypothesize that $1,25(OH)_2D_3$ may exert its activity in two mechanisms—a direct effect on the cytokine message (transcription and/or stability), and an indirect effect via enhancement of LPS binding and thus activity. Our candidate for modulating LPS activity is CD14. The CD14 protein has been shown to function as a high-affinity cell surface receptor molecule for LPS. In Figure 3A we show that $1,25(OH)_2D_3$ increases CD14 mRNA abundance. Already at 24 h of incubation a significant difference is observed between BMMs incubated in the absence or presence of $1,25(OH)_2D_3$. Maximal effect is observed when BMMs were incubated with the hormone for 72 h (> 2-fold increase). In Figure 3B we show the combined effect of $1,25(OH)_2D_3$ and LPS.

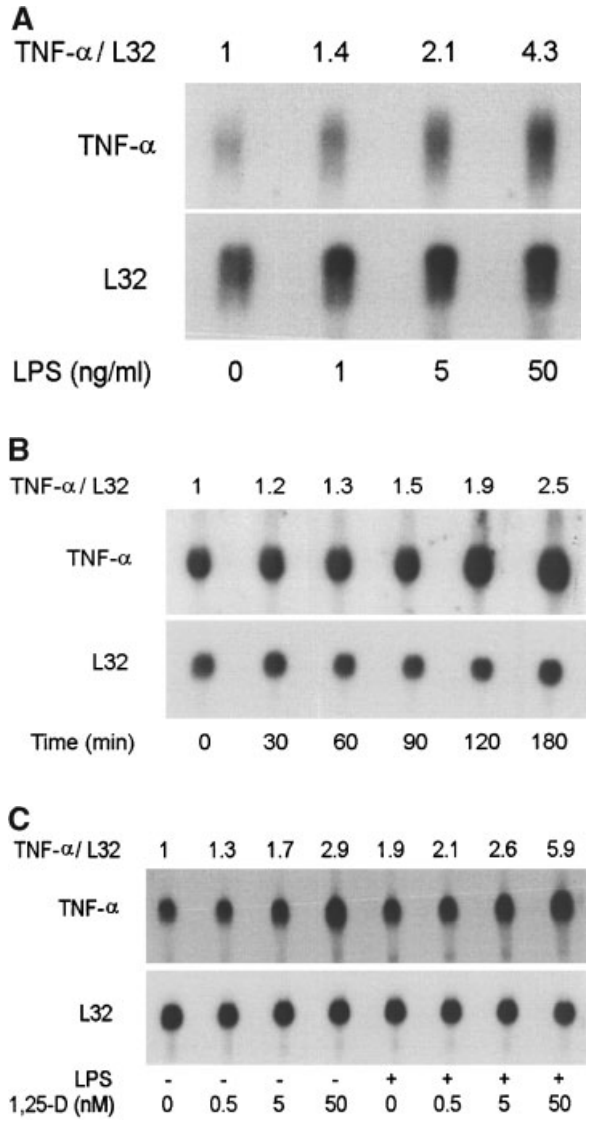

Fig. 2. Modulation of TNF-α mRNA abundance in BMMs by $1,25(OH)_2D_3$ and LPS. **A**: Cells were incubated for 72 h, followed by activation with different doses of LPS for 2 h; (**B**) cells were incubated as in A and LPS (5 ng/ml) was added for different time points. **C**: Cells were incubated in the presence of different doses of $1,25(OH)_2D_3$ for 72 h with or without LPS (5 ng/ml, last 2 h). Transcript abundance was measured as described in Figure 1.

Increases of 120% and 80% in CD14 mRNA abundance are observed with $1,25(OH)_2D_3$ (50 nM) and LPS (5 ng/ml), respectively. The combination of $1,25(OH)_2D_3$ and LPS caused 270% increase.

Next, we compared TNF-α and CD14 transcript abundance between vitamin D depleted (−D) and repleted mice (+D) (Fig. 4). Abundance of TNF-α and CD14 transcript is higher (~60% and ~67%, respectively) in +D BMMs than in −D BMMs. The increase in transcript

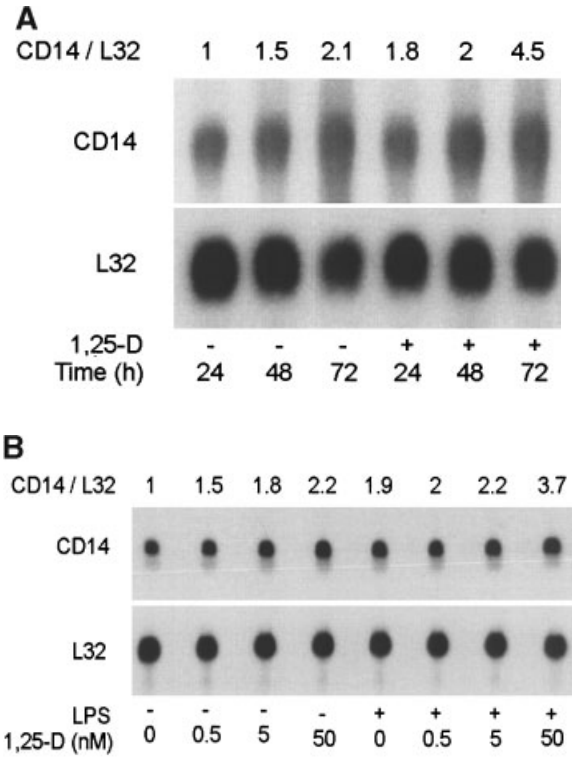

Fig. 3. Modulation of CD14 mRNA abundance in BMMs by $1,25(OH)_2D_3$ and LPS. **A**: Cells were incubated in the presence or absence of $1,25(OH)_2D_3$ (50 nM) for the indicated time. **B**: Cells were incubated in the presence of different doses of $1,25(OH)_2D_3$ for 72 h, with or without LPS (5 ng/ml, last 2 h). CD14 transcript abundance was measured.

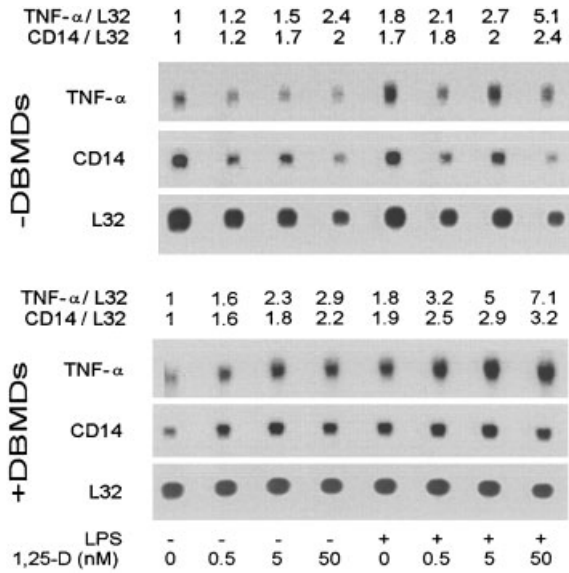

Fig. 4. Modulation of TNF-α and CD14 mRNA abundance by $1,25(OH)_2D_3$ in vitamin D depleted (−D) and repleted (+D) mice. BMMs derived from −D (top half) and +D (bottom half) mice were incubated for 72 h in the presence of different doses of $1,25(OH)_2D_3$, followed by activation with LPS (5 ng/ml, 2 h). TNF-α and CD14 transcript abundance were measured.

abundance of both genes by $1,25(OH)_2D_3$ and LPS was slightly more pronounced in cells derived from the repleted mice. Addition of $1,25(OH)_2D_3$ at 50 nM caused an increase of 140% and 190% in TNF-α mRNA levels in −D and +D derived cells, respectively. LPS alone, increased transcript abundance by 80% in both types of cells. The combination of the two modulators increased the basal levels of TNF-α mRNA by 410% and 610% in −D and +D derived cells, respectively, indicating a positive synergy. Addition of $1,25(OH)_2D_3$ at 50 nM increased CD14 mRNA levels in −D and +D derived cells to a similar degree (100% and 120%, respectively). LPS alone (5 ng/ml) increased CD14 mRNA levels in the two types of cells by 70% and 90%, respectively. The addition of the two modulators increased CD14 mRNA level by 140% and 220% in −D and +D derived cells, respectively.

In contrast to $1,25(OH)_2D_3$, addition of 9-*cis* retinoic acid (9cRA) to BMMs caused a marked reduction (~5-fold) in the abundance of TNF-α mRNA (Fig. 5A). The combined treatment of BMMs with 9cRA and LPS resulted in a marked decrease (~2-fold) in TNF-α mRNA levels, although LPS alone increased TNF-α mRNA levels by 80% in BMMs. In contrast to the inhibitory effect of 9cRA observed on TNF-α mRNA, the retinoid caused a marked increase in CD14 mRNA (up to ~3-fold increase) in a dose dependent manner. LPS augmented only slightly the retinoid effect on CD14 mRNA (Fig. 5A). Treatment of BMMs with $1,25(OH)_2D_3$ or 9cRA (50 nM each), results in increased levels of CD14 protein by 290% and 370%, respectively, as demonstrated in Western blotting analysis (Fig. 5B).

In Figure 6A we show that when BMMs were incubated with the retinoid and the vitamin D metabolite together, the inhibitory effect of 9cRA dominated the increase in TNF-α mRNA obtained with $1,25(OH)_2D_3$. For example, $1,25(OH)_2D_3$ (50 nM) caused 160% increase, 9cRA (50 nM) caused 65% decrease, and when added together, 20% decrease was recorded. Analyses of CD14 mRNA levels show that 9cRA enhances the effect of $1,25(OH)_2D_3$. In Figure 6B we see the effect of the three modulators when added together on TNF-α mRNA abundance. We see that 9cRA not only reversed the increased TNF-α mRNA abundance observed

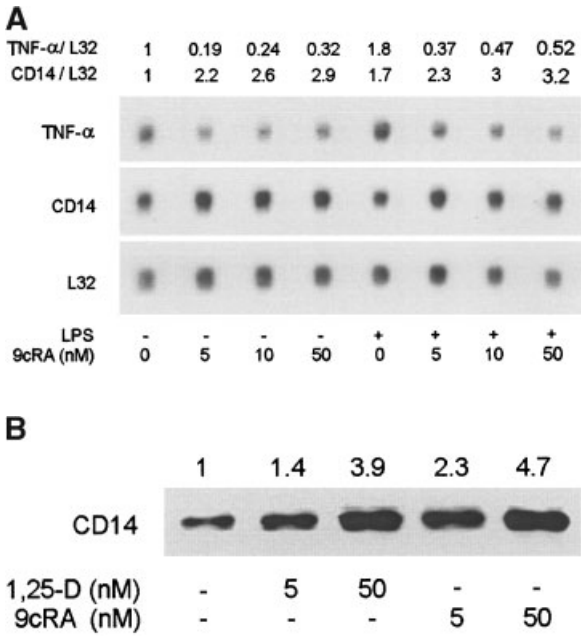

Fig. 5. Modulation of TNF-α mRNA and CD14 mRNA and protein abundance in BMMs by 9cRA. **A**: Cells were incubated in the presence or absence of different doses of 9cRA for 72 h, with or without LPS activation (5 ng/ml, last 2 h). TNF-α and CD14 transcript abundance were measured. **B**: Cells were incubated in the presence of $1,25(OH)_2D_3$ or 9cRA for 72 h. Western analysis was performed using anti-CD14 antibodies.

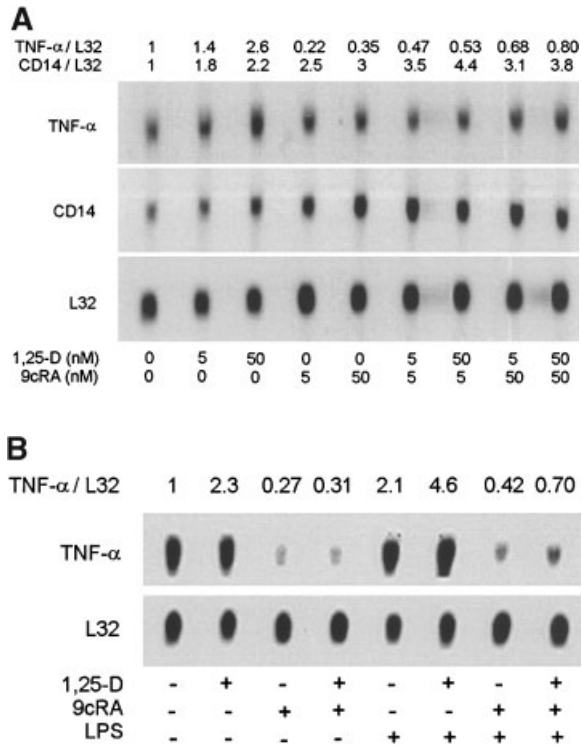

Fig. 6. Modulation of TNF-α and CD14 mRNA abundance in BMMs by $1,25(OH)_2D_3$, 9cRA and LPS. **A**: Cells were incubated for 72 h with $1,25(OH)_2D_3$ or 9cRA or their combinations. TNF-α and CD14 transcript abundance were measured. **B**: BMMs were incubated with $1,25(OH)_2D_3$ (50 nM) or 9cRA (50 nM) or both for 72 h, with or without LPS activation (5 ng/ml, last 2 h). TNF-α transcript abundance was measured.

with $1,25(OH)_2D_3$ and LPS when incubated with each, but also was able to overcome the increase when BMMs treated with both, $1,25(OH)_2D_3$ and LPS.

To gain insight into the mechanism of $1,25(OH)_2D_3$ and 9cRA modulation of TNF-α mRNA abundance in BMMs, we examined their effects on the stability of the cytokine mRNA. Transcript half-life was measured by adding actinomycin D, a potent inhibitor of RNA polymerase II-dependent transcription, and Northern analyses at various time points with the inhibitor. Actinomycin-D (2.5 μg/ml), was added to unstimulated BMMs or cells stimulated with $1,25(OH)_2D_3$, 9cRA, or LPS. Steady-state levels of TNF-α and L32 mRNA were measured at the indicated time points, using Northern blot analysis (Fig. 7A). Figure 7B shows the average TNF-α/L32 mRNA ratio derived from three independent experiments. As expected, TNF-α mRNA levels measured in cells treated with either $1,25(OH)_2D_3$ or LPS were higher than in untreated BMMs while 9cRA-treated BMMs expressed lower transcript level. TNF-α mRNA half-life in unstimulated BMMs was 18 ± 0.6 min. No significant change was found in cells treated with either $1,25(OH)_2D_3$ or LPS (half-life of 17 ± 0.4 and 20 ± 1.1 min, respectively). Addition of 9cRA shortened the half-life of the cytokine message to 12 ± 0.8 min ($P < 0.002$).

To examine the possibility of transcriptional regulation of TNF-α by $1,25(OH)_2D_3$, we first searched for potential VDREs in the promoter of the cytokine gene (Table I).

Using EMSA we have examined binding activity of nuclear proteins prepared from BMMs treated for 3 days with $1,25(OH)_2D_3$ to the putative VDREs. Each of the six potential VDREs was labeled, and incubated with the nuclear extract in the absence or presence of 100-fold unlabeled corresponding oligodeoxynucleotide sequence. In Figure 8 we see significant binding activity only with one of the tested oligonucleotides, (−1008 to −994, termed tnfVDRE).

Nuclear-extract binding of tnfVDRE is modulated by $1,25(OH)_2D_3$ and 9cRA. In Figure 9 we see the activity of nuclear extracts of untreated

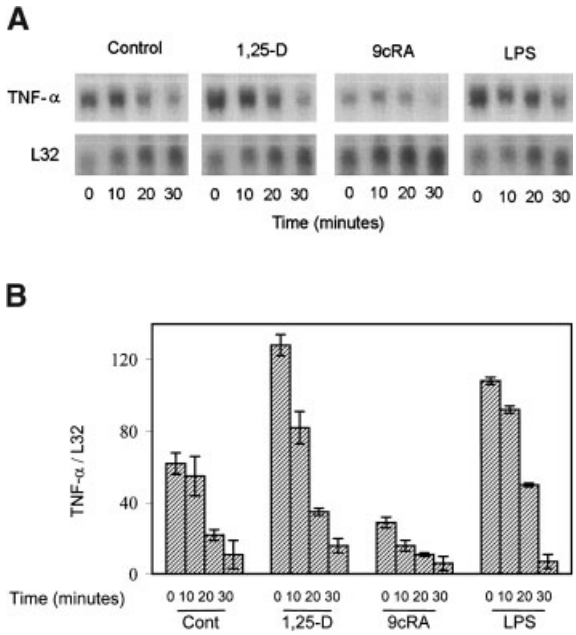

Fig. 7. Modulation of TNF-α mRNA stability in BMMs. Cells were incubated in the presence of either $1,25(OH)_2D_3$ (50 nM) or 9cRA (50 nM) for 72 h. Alternatively, cells were incubated for 72 h in the absence of either modulator and activated with LPS (5 ng/ml, last 2 h). Then actinomycin D was added for the indicated time points. TNF-α mRNA abundance was examined. **A**: A representative autoradiogram. **B**: Densitometric analysis (average of three experiments).

BMMs and cells treated with $1,25(OH)_2D_3$, 9cRA, or both. $1,25(OH)_2D_3$ increases the binding activity by ~4.5-fold. 9cRA augments the binding activity of $1,25(OH)_2D_3$ (~7-fold as compared to untreated cells), but it does not affect the binding in the absence of the vitamin

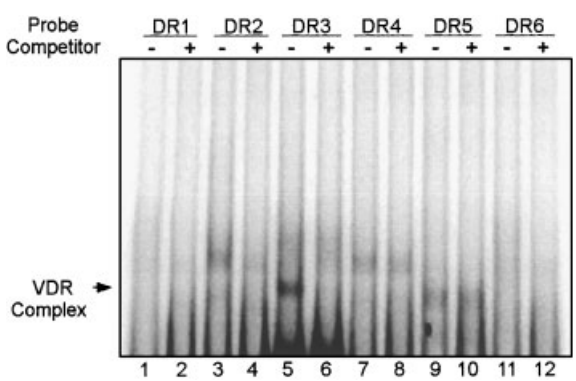

Fig. 8. EMSA of putative VDREs in TNF-α promoter. BMMs were incubated for 72 h and nuclear extracts were prepared. Binding reactions were performed with the various ^{32}P-labeled putative VDREs in the absence (odd lanes) or presence (even lanes) of 100-fold molar excess of the corresponding unlabeled oligonucleotides.

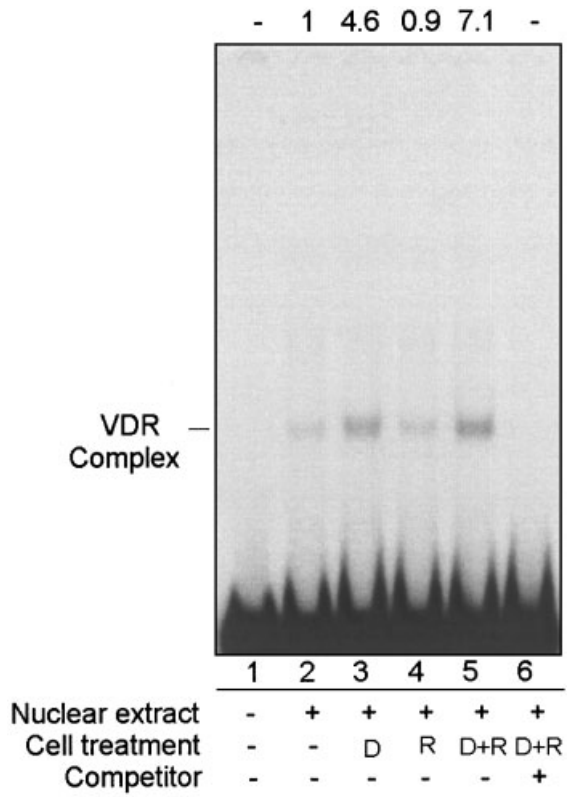

Fig. 9. Modulation of the nuclear-extract/tnfVDRE binding by $1,25(OH)_2D_3$ and 9cRA. BMMs were incubated for 72 h in the absence or presence of $1,25(OH)_2D_3$ (50 nM), 9cRA (50 nM), or both. Nuclear extracts prepared from the cells, and ^{32}P-labeled tnfVDRE were used for EMSA. A representative autoradiogram is shown.

D metabolite. Treatment of cells with either the retinoid and/or the vitamin D metabolite did not increase binding activity of nuclear extract to the other putative TNF-α promoter derived VDREs (not shown).

We next compared the ability of the various putative VDREs found in TNF-α promoter to compete with tnfVDRE. In this experiment, nuclear extracts were incubated with labeled tnfVDRE in the absence or presence of the unlabeled sequences (10, 25, 50, and 100-fold excess). Unlabeled tnfVDRE was the most effective inhibitor (~76% inhibition at 10-fold excess), and DR6 did not affect the binding (<5% inhibition at 10-fold) (Fig. 10A,B). The other sequences exhibited intermediate degrees of competition of tnfVDRE binding (at 10-fold excess DR1, DR2, DR4, and DR5 caused inhibition of 47, 40, 43, and 23%, respectively).

The ability of previously characterized VDREs to compete with tnfVDRE binding was

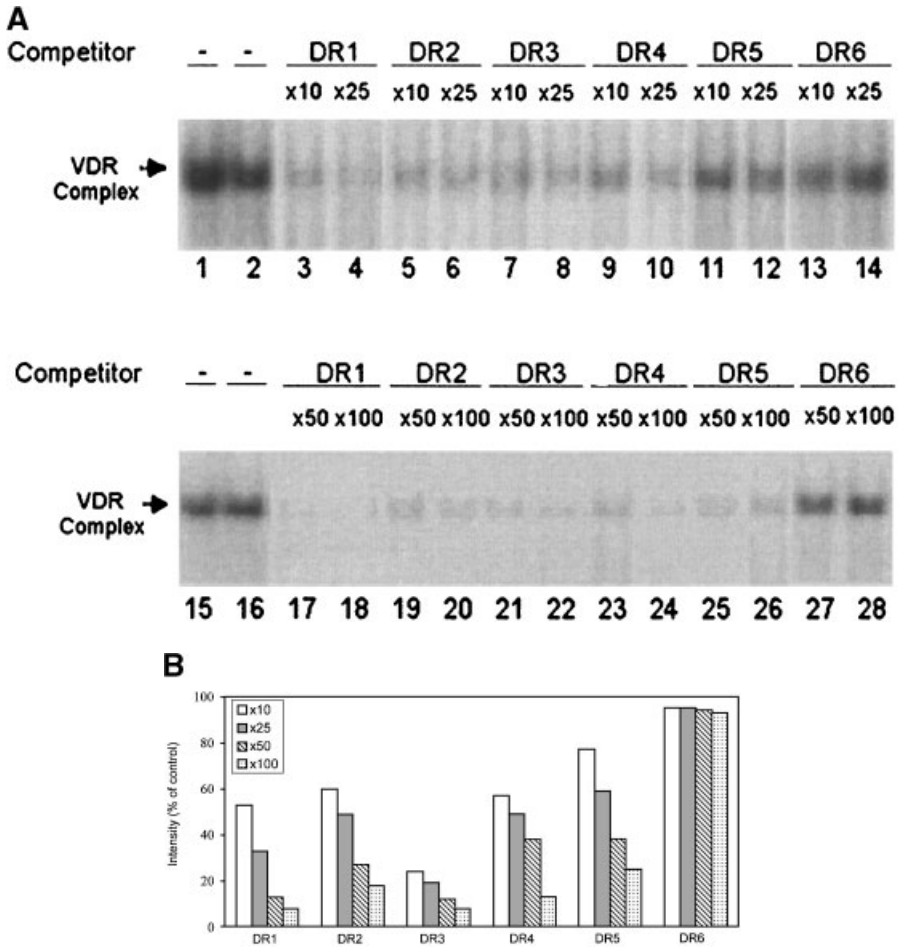

Fig. 10. Competition analyses of the putative TNF-α promoter derived VDREs. Cells were incubated with $1,25(OH)_2D_3$ (50 nM) and 9cRA (50 nM) for 72 h. EMSA using nuclear extracts derived from these cells and ^{32}P-labeled tnfVDRE was performed in the absence of competitors (**lanes 1**, **2**, **15**, **16**) or in the presence of 10- and 25-fold excess of competitors (**lanes 3–14**), or in the presence of 50- and 100-fold excess of competitors (**lanes 17–28**). A representative autoradiogram (**A**) and a densitometric analysis (**B**) are shown.

examined. We have used a sequence from mouse osteopontin promoter (opVDRE) [Breen et al., 1994] and a sequence from rat osteocalcin (ocVDRE) [Markose et al., 1990]. We have also used a mutated sequence of ocVDRE (SHEmt) [Staal et al., 1996]. In this experiment, nuclear extracts were incubated with labeled tnfVDRE in the absence or presence of the unlabeled oligodeoxynucleotides (25, 50, and 100-fold excess). We see in Figure 11 that the most effective inhibitor is the unlabeled tnfVDRE (84% inhibition at 50-fold excess). opVDRE was more effective than ocVDRE (35% and 22% inhibition at 50-fold excess, respectively). Indeed there is a higher degree of sequence homology between tnfVDRE and opVDRE than between tnfVDRE and ocVDRE. SHEmt did not compete with the binding (~7% inhibition at 50-fold excess).

In order to determine the protein composition of the tnfVDRE-bound complex we examined the effects of anti-VDR and anti-RXR antibodies on the migration of the complex (Fig. 12). The complex was super shifted when antibodies recognizing the ligand-binding domain of either VDR or RXR were included. Monoclonal antibodies recognizing the DNA binding domains of either VDR or RXR inhibited the binding of the nuclear extract to labeled tnfVDRE.

DISCUSSION

We have previously shown that $1,25(OH)_2D_3$ induces the synthesis of TNF-α. Consistent with the in vitro findings, $1,25(OH)_2D_3$ was also found to modulate TNF-α production in vivo, and the circulating cytokine levels in LPS

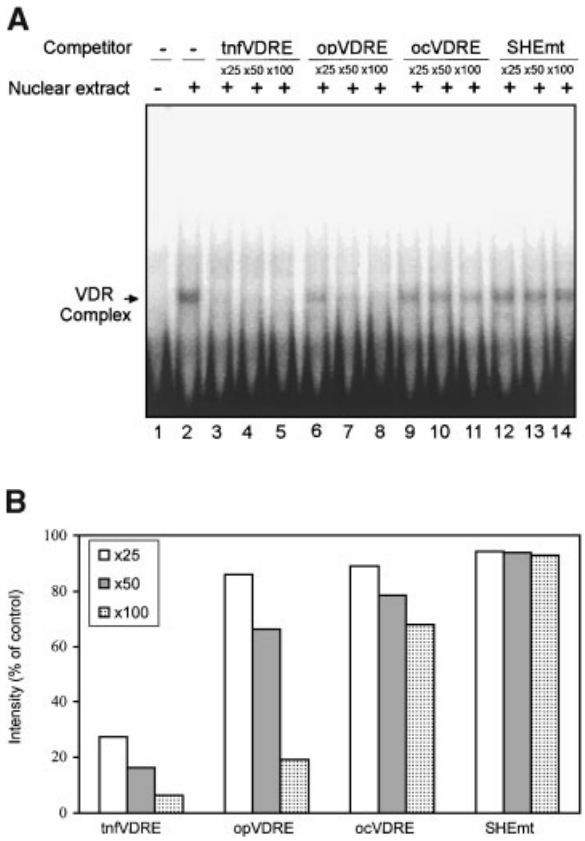

Fig. 11. Competition analyses of tnfVDRE with established VDREs. Nuclear extracts were prepared as in Figures 9 and 10. EMSA was performed using labeled tnfVDRE. Competitors included opVDRE, ocVDRE and SHEmt at 25, 50, and 100-fold excess. A representative autoradiogram (**A**) and a densitometric analysis (**B**) are shown.

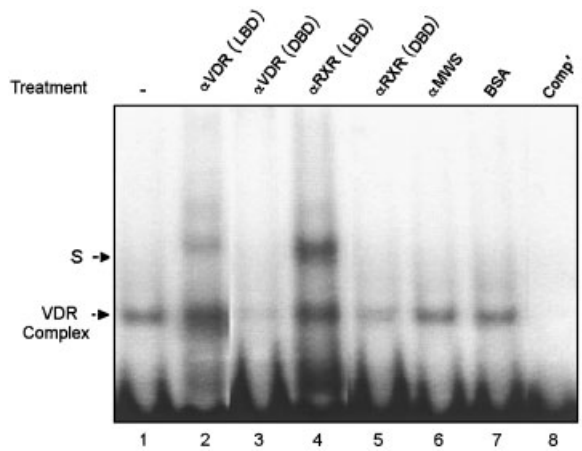

Fig. 12. The VDR-complex formed with the tnfVDRE is composed of a VDR/RXR heterodimer. EMSA was performed as described in the previous figures. Nuclear extract was treated with anti-VDR antibody (recognizing the ligand binding domain, **lane 2**, and the DNA binding domain, **lane 3**), anti-RXR antibody (recognizing the ligand binding domain, **lane 4**, and the DNA binding domain, **lane 5**), anti-mouse whole serum (**lane 6**). Control nuclear extracts in the absence or presence of BSA is shown in **lanes 1** and **7**, respectively, and competition with 100-fold excess of unlabeled tnfVDRE is shown in **lane 8**.

injected mice significantly higher in +D as compared to −D mice [Kankova et al., 1991; Abu-Amer and Bar-Shavit, 1993].

Studies on modulation of TNF-α by $1,25(OH)_2D_3$ yielded contradictory results. $1,25(OH)_2D_3$ increased TNF-α production in LPS-stimulated U937 cells (a human myelomonocytic cell line) [Fagan et al., 1991; Prehrn et al., 1992]. In contrast, $1,25(OH)_2D_3$ inhibited the LPS-induced production of TNF-α in isolated mature peripheral blood monocytes [Riancho et al., 1993; Panichi et al., 1998; Giovannini et al., 2001]. Inhibitory effect was also observed with human peritoneal macrophages from patients on continuous ambulatory peritoneal dialysis [Cohen et al., 2001; Shany et al., 2001]. It seems that that the differentiation/maturation status of the cells plays a role in determining their response to $1,25(OH)_2D_3$. The hormone increases TNF-α production in immature cells (cell-lines, bone marrow cells) and decreases the cytokine in more mature (peripheral blood monocytes and peritoneal macrophages).

In the present study, we found that the steady state level of TNF-α mRNA is increased by $1,25(OH)_2D_3$. Furthermore, the addition of $1,25(OH)_2D_3$ together with LPS, resulted in a positive synergistic effect.

CD14 and TLR-4 are required for the effects of low concentration of LPS (1–10 ng/ml) [Kirschning et al., 1998; Tsan et al., 2001]. The lack of CD14, due to either the use of CD14 knockout mice, or removal of CD14 with phospholipase C, results in a complete block in TNF-α production [Haziot et al., 1996]. We find that already at low LPS concentrations (1 and 5 ng/ml), 40% and 100% increase in TNF-α mRNA was observed, respectively. Our results prompted us to hypothesize that $1,25(OH)_2D_3$ may exert its activity in two mechanisms: a direct effect on the cytokine message (rate of transcription and/or stability), and indirect effect via enhancement of LPS activity. Induction of differentiation in the human myeloid cell line, HL-60, by $1,25(OH)_2D_3$ is accompanied by a marked increase in CD14 expression, while TLR-4 expression is almost unaffected [Li and Stashenko, 1992].

In the present study, we find that $1,25(OH)_2D_3$ increases the CD14 mRNA and

protein abundance in BMMs. The hormone-induced increase in CD14 expression could modulate the binding and activity of LPS.

The in vivo significance of the findings was confirmed by studying modulation of TNF-α and CD14 message abundance between −D and +D mice. $1,25(OH)_2D_3$, LPS and their combination affected in a similar manner the abundance of TNF-α and CD14 mRNA in −D and +D derived cells. The effects, however, were slightly more pronounced in +D-derived cells. Furthermore, basal TNF-α and CD14 transcript abundance was higher in +D than in −D derived cells (Fig. 4).

Nuclear hormone receptors, including VDR, act either to activate or to repress transcription by binding to response elements in the promoter region of target genes. In addition to natural DR3-type response elements, DR4-, DR6-, IP7-, and IP9-type structures have been recognized as candidate VDREs [Carlberg, 1993; Schrader et al., 1994; Wu et al., 1999]. The binding of $1,25(OH)_2D_3$ to VDR alters its conformation to promote heterodimerization with the RXR, and association of the heterodimer complex with the VDRE [Yu et al., 1991; Zou et al., 1997].

We find that $1,25(OH)_2D_3$ increases transcript levels of TNF-α and CD14 in BMMs. On the other hand, 9cRA decreases TNF-α mRNA levels, and increases the CD14 mRNA levels in these cells. Furthermore, the inhibitory effect of 9cRA dominated the stimulatory effects of $1,25(OH)_2D_3$ and LPS. Thus, 9cRA utilizes a different mechanism(s) to modulate TNF-α and CD14.

To gain insight into the mechanism of $1,25(OH)_2D_3$ and 9cRA modulation of TNF-α mRNA abundance in BMMs, we examined their effect on TNF-α mRNA stability. TNF-α mRNA rate of degradation was not affected by the presence of either $1,25(OH)_2D_3$ or LPS. On the other hand, addition of 9cRA shortened TNF-α mRNA half-life. Thus, it is unlikely that the increase in TNF-α mRNA abundance caused by $1,25(OH)_2D_3$ and LPS involves modulation of transcription stability. The decrease in TNF-α mRNA abundance caused by 9cRA, could involve enhanced TNF-α mRNA degradation. These findings are consistent with recent report showing that TNF-α mRNA stability was not affected by LPS, and that retinoic acid induced destabilization of the cytokine transcripts in hepatic macrophages [Raabe et al., 1998].

In order to examine the involvement of transcriptional regulation of TNF-α by $1,25(OH)_2D_3$, we screened the promoter region of the murine TNF-α gene [Semon et al., 1987; Iraqi and Teale, 1997] for consensus VDREs, and obtained six putative sequences (Table I). We identified out of these sequences a functional VDRE between positions −1008 and −994, (tnfVDRE).

Incubation of BMMs with $1,25(OH)_2D_3$ increased the nuclear extract binding to the tnfVDRE. Cell treatment with 9cRA did not have any effect on the binding activity, but markedly increased the activity of $1,25(OH)_2D_3$.

The oligodeoxynucleotides sequence play an important role in the affinity of their interactions with the VDR-complex [Forman et al., 1992; Schrader et al., 1995]. For the competition analyses we chose VDREs derived from mouse osteopontin (opVDRE) and rat osteocalcin (ocVDRE) promoters. opVDRE represents a perfect DR of two similar steroid half-sites (GGTTCA-CGA-GGTTCA) while ocVDRE represents an imperfect DR of two distinct steroid half sites (GGGTGA-ATG-AGGACA). A mutated non-active sequence of the ocVDRE (SHEmt) was also used (GtaTGA-ATG-ActACA). opVDRE competed with tnfVDRE binding more efficiently than ocVDRE. The higher degree of sequence homology between the tnfVDRE and opVDRE (10/15), than the tnfVDRE and ocVDRE (6/15) is consistent with our finding.

The effects of anti-VDR and anti-RXR antibodies on the interactions between BMMs-derived nuclear extracts and tnfVDRE revealed that the complex contains VDR/RXR heterodimers. Surprisingly, the presence of the monoclonal anti-VDR and anti-RXR antibodies caused an increase in original band intensity. Similar phenomenon was observed previously [Staal et al., 1996]. They offered the explanation, that enhancement of complex binding to VDRE is due to nonspecific effects of the antibodies, that could alter the binding equilibrium.

Although 9cRA decreases TNF-α mRNA levels in $1,25(OH)_2D_3$ treated cells, a more efficient binding activity is observed in nuclear extract derived from cells treated with both modulators than in nuclear extract derived from $1,25(OH)_2D_3$ treated cell (Fig. 9). 9cRA may exert its effect on TNF-α mRNA in different ways: (1) by decreasing the stability of the mRNA transcripts (post-transcriptional modulation), as shown in Figure 7, and/or (2)

by inhibiting the activity of the transcription complex (transcriptional modulation).

Retinoid receptors indeed were shown to employ various mechanisms in post-transcriptional regulation. RAR complex has been shown to stabilize transcripts of prolactin in IM-9-IP3 cells (B-lymphoblast cell line) [Gellersen et al., 1992], or CRABP-II in F9-teratocarcinoma cells [MacGregor et al., 1992]. On the other hand, retinoids suppress the expression of adipsin in 3T3-F442A (adipocytic cell line) by decreasing the transcripts stability, probably via induction or activation of specific nuclease activity [Antras et al., 1991]. In addition, in the case of cytochrome P-450d, it was reported that direct interaction between the nuclear receptor and the RNA precursors might occur. In this case it may induce a more favorable structure for efficient splicing or protection of the molecule from rapid nuclear degradation [Silver and Krauter, 1990]. Retinoid addition to LPS-stimulated rat hepatic macrophages caused a marked inhibition in TNF-α expression probably via decreasing its message stability [Silver and Krauter, 1990]. Our results regarding modulation of TNF-α mRNA abundance by the VDR and RXR ligands are consistent with a recent report [Jimenez-Lara and Aranda, 2000] suggesting that VDR/RXR heterodimers could bind VDRE in a transcriptionally unproductive manner.

The present study strengthens the notion that $1,25(OH)_2D_3$, in addition to its central role in calcium homeostasis, is also a physiological modulator of macrophage function. We show that $1,25(OH)_2D_3$ increases TNF-α transcript abundance in BMMs via a direct transcriptional mechanism, in which a VDR-RXR heterodimer complex is involved. Moreover, TNF-α mRNA levels are further enhanced via an indirect mechanism, in which LPS effect is involved. 9cRA probably employs transcriptional and post-transcriptional mechanisms to decrease the cytokine message abundance.

ACKNOWLEDGMENTS

This research was supported by a grant from ICRF. We thank Harry Schwartz for technical assistance.

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