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Regulation of interleukin-1 and tumour necrosis factor gene expression in myelomonocytic cell lines by 1,25-dihydroxyvitamin D_3

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SUMMARY

1,25-dihydroxyvitamin D_3[1,25-$(OH)_2D_3$] is capable of regulating cells in the immune system and affects cytokine production by both T lymphocytes and by monocytes. We examined the effects of 1,25-$(OH)_2D_3$ on the regulation of interleukin-1β (IL-1β) and tumour necrosis factor-alpha (TNF-α) genes in HL-60 and U937 cells. 1,25-$(OH)_2D_3$ alone only induced low level expression of the genes for these cytokines. Phorbol 12-myristate 13-acetate (PMA) strongly induced the transcription of these genes, whilst the addition of 1,25-$(OH)_2D_3$ to PMA-stimulated cells caused a further dose-dependent synergistic increase in the mRNA for both cytokines in U937 cells. In PMA-stimulated HL-60 cells, 1,25-$(OH)_2D_3$ increased the mRNA for IL-1β but not that for TNA-α, These differences may be related to the different stage of myeloid differentiation in HL-60 and U937 cells.

INTRODUCTION

The active metabolite of vitamin D_3, 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$], behaves like a hormone and plays an essential role in mineral homeostasis. Specific, high affinity receptors for 1,25-$(OH)_2D_3$ are present in a variety of cell types other than the classical target tissues of bone, intestine and kidney. These receptors occur in activated, but not resting, T and B lymphocytes, in monocytes and in myeloid cell lines such as U937 and HL-60.[1,2] The hormone has been shown to down-regulate interleukin-2 (IL-2), interferon-gamma (IFN-γ) and granulocyte- macrophage colony-stimulating factor GM-CSF production in T lymphocytes.[3-6] 1,25-$(OH)_2D_3$ induces the differentiation of myeloid cell lines such as U937 and HL-60 along the monocyte pathway.[7-10] In addition, the hormone alters monocyte/macrophage functions such as oxidative metabolism,[11] increases IL-1 production by freshly isolated human monocytes[4] and augments IL-1 production induced in U937 cells by a factor produced by human T lymphocytes.[8] We have examined the effects of 1,25-$(OH)_2D_3$ on the transcription of IL-1β and tumour necrosis factor-alpha (TNF-α) genes in myeloid cell lines. Whilst 1,25-$(OH)_2D_3$ alone increased expression of the gene for IL-1β in both HL-60 and U937 cell lines, it did not appear to increase expression of the TNF-α gene in either of these cell lines. However, once transcription had been activated by phorbol 12-myristate 13-acetate (PMA) 1,25-$(OH)_2D_3$ enhanced the level of the mRNA for both IL-1β and TNF-α in U937 cells, but only for IL-1β in HL-60 cells.

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MATERIALS AND METHODS

Reagents

1,25-$(OH)_2D_3$ was a kind gift from Dr Uskokovich, Hoffman-La Roche, Nutley, NJ. The metabolite was dissolved in 100% ethanol. PMA, lipopolysaccharide (LPS; from *Escherichia coli*), and polymyxin B sulphate were obtained from Sigma Chemical Company, Poole, Dorset, U.K. PMA was dissolved in dimethyl sulphoxide (DMSO). LPS and polymyxin B sulphate were dissolved in phosphate-buffered saline (PBS). The final concentration of ethanol/DMSO in culture did not exceed 0·1%. RPMI-1640 tissue culture medium and foetal calf serum (FCS) were obtained from Life Technologies Ltd, Paisley, Renfrewshire, U.K.

Cells and culture conditions

U937 and HL-60 cells were maintained in RPMI-1640 tissue culture medium supplemented with 10% FCS, L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml). The cell lines were screened for mycoplasma contamination using the Hoechst stain method (ICN-Flow Laboratories) and found to be negative. Cells in log phase were incubated in the presence of either 10 ng/ml PMA, 1 μg/ml LPS, or 10^{-6}–10^{-10} M 1,25-$(OH)_2D_3$. The following conditions were also included: PMA + LPS, PMA + 1,25-$(OH)_2D_3$, LPS + 1,25-$(OH)_2D_3$ and PMA + LPS + 1,25-$(OH)_2D_3$. In cultures where LPS was absent, polymyxin B sulphate was used to inhibit any contaminating LPS present in the tissue culture medium. Controls were included containing the corresponding amounts of DMSO, ethanol and polymyxin B sulphate. These substances had no effect on the modulation of cytokine mRNA. Cell cultures were harvested after 18 hr, a period previously shown by Steffen *et al.* to be near

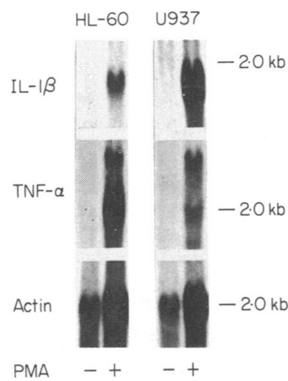

Figure 1. Northern blot analysis of IL-1β and TNF-α mRNA expression with and without PMA stimulation in HL-60 and U937 cells. The same filter was also hybridized with chicken β-actin to determine the amount of RNA loaded.

optimal for TNF-α mRNA induction in HL-60 cells.[12] Cell viability, as assessed by staining with ethidium bromide/acridine orange and fluorescence microscopy, was always greate than 95%.

RNA isolation, Northern blot and slot blot analysis

Total cellular RNA was isolated by guanidium isothiocyanate lysis and caesium chloride density-gradient centrifugation, modified from Chirgwin *et al.*[13] For Northern blots, 10–20 μg of total RNA were analysed by electrophoresis in 1·0% agarose-formaldehyde gels, followed by Northern blot transfer to nylon membranes (Hybond-N, Amersham, Bucks, U.K.). For slot blot analysis 5 μg of total RNA were used.

Blots were prehybridized at 42° for 2–4 hr in 50% formaldehyde, 5 × SSPE (0·9 M sodium chloride, 50 mM sodium phosphate pH 7·7, 0·5 mM EDTA), 5 × Denhardt's solution (0·1% Ficoll, 0·1% bovine serum albumin fraction V, 0·1% polyvinylpyrrolidone), 0·5% SDS and 50 μg/ml sonicated salmon sperm DNA. Hybridization was carried out in the same solution for 16 hr at 42° with ^{32}P-dCTP-labelled 2·8 kb human TNF-α DNA[14] or 0·53 kb human IL-1β cDNa.[15] Chicken β-actin cDNA was used as a control probe. Probes were labelled using the multiprime labelling kit (Amersham International, Amersham, Bucks, U.K.). After hybridization the blots were washed twice in 2 × SSPE, 0·1% SDS at 42° for 15 min, once in 1 × SSPE, 0·1% SDS at 42° for 30 min, and finally twice in 0·1 × SSPE, 0·1% SDS at room temperature for 15 min. The RNA blots were then exposed to Hyperfilm-MP (Amersham International) with intensifying screens at −70° for 3–7 days. The intensity of each slot on the autoradiographs was assessed by scanning densitometry.

RESULTS

Effect of 1,25-$(OH)_2D_3$ on PMA-induced IL-1β and TNF-α mRNA in HL-60 cells

Messenger RNA for IL-1β and TNF-α in unstimulated HL-60 cells was either undetectable or only present at a low basal level (Fig. 1). LPS alone did not enhance IL-1β or TNF-α mRNA transcription in these cells nor did it influence the response to the other agents tested (data now shown). The addition of PMA to HL-60 cells results in the induction of IL-1β and TNF-α mRNA, as shown by Northern blot analyses (Fig. 1). Slot blot analyses of total RNA confirmed this effect and also showed that, in cells treated with 1,25-$(OH)_2D_3$ alone there was enhanced expression of mRNA for IL-1β but not for TNF-α (Fig. 2). The addition of PMA and 1,25-$(OH)_2D_3$ to HL-60 cells resulted in an augmentation of IL-1β but not TNF-α mRNA expression (Fig. 2). This increase was dose-dependent over a range of 10^{-10}–10^{-6} M 1,25-$(OH)_2D_3$, as shown in three slot blot experiments (Fig. 3). Both the induction and augmentation of mRNA that was observed is specific for the cytokines and not the result of a general increase in mRNA since β-actin binding remained constant under all experimental conditions.

Effect of 1,25-$(OH)_2D_3$ on PMA induced IL-1β and TNF-α mRNA in U937 cells

Northern blot analyses demonstrated that in unstimulated U937 cells the basal levels of transcription of the genes for IL-1β and TNF-α is low (Fig. 1). Exposure to PMA for 18 hr greatly increased the specific expression of the mRNA for these two cytokines (Fig. 1). 1,25-$(OH)_2D_3$ alone failed to have any effect on the transcription of the TNF-α gene and only had a slight effect on IL-1β gene expression (Fig. 2). However, the amount of mRNA for these cytokines that is induced by PMA was augmented by 1,25-$(OH)_2D_3$ (Fig. 2). That 1,25-$(OH)_2D_3$ is able to further augment PMA-induced transcription of the genes for IL-1β and TNF-α in a dose-dependent manner was confirmed in further experiments shown in Fig. 3. Unlike the situation with HL-60 cells, 1,25-$(OH)_2D_3$ increased the mRNA for both IL-1β and TNF-α.

DISCUSSION

The results of this study show that the physiologically active metabolite of vitamin D, 1,25-$(OH)_2D_3$, is capable of augmenting PMA-induced transcription of the genes for IL-1β and TNF-α in myeloid cell lines. 1,25-$(OH)_2D_3$ alone had no effect on TNF-α gene expression and only relatively small effects on IL-1β gene expression, consistent with an earlier report which showed that the hormone failed to induce IL-1 production by U937 cells but was able to augment the production of IL-1 once it had been initiated by mitogen.[8] More recently, Ucla *et al.*[16] have shown that preincubation of U937 cells with 1,25-$(OH)_2D_3$ increased the IL-1β mRNA response to LPS in a subline of U937 which was already responsive to LPS alone. These authors examined different sublines of U937 obtained from different laboratories and found that they exhibited differing properties, for example the presence or absence of an IL-1β mRNA response to LPS stimulation. As indicated, our U937 cell line was not responsive to LPS.

Some differences in the action of 1,25-$(OH)_2D_3$ were observed between U937 and HL-60 cells. In PMA-stimulated U937 cells the hormone further increased transcription of both cytokine genes but in HL-60 this effect was restricted, at least when measuring mRNA levels 18 hr post-stimulation, to the IL-1β gene. We cannot, however, exclude the possibility that additional effects on cytokine mRNA levels might be observed after different times of stimulation with 1,25-$(OH)_2D_3$. Nevertheless, our results suggest that the responsiveness of myeloid cells to 1,25-$(OH)_2D_3$ may be governed by their state of differentiation. U937 cells, arrested at a later stage of differentiation than HL-60 cells, are already committed to the monocyte

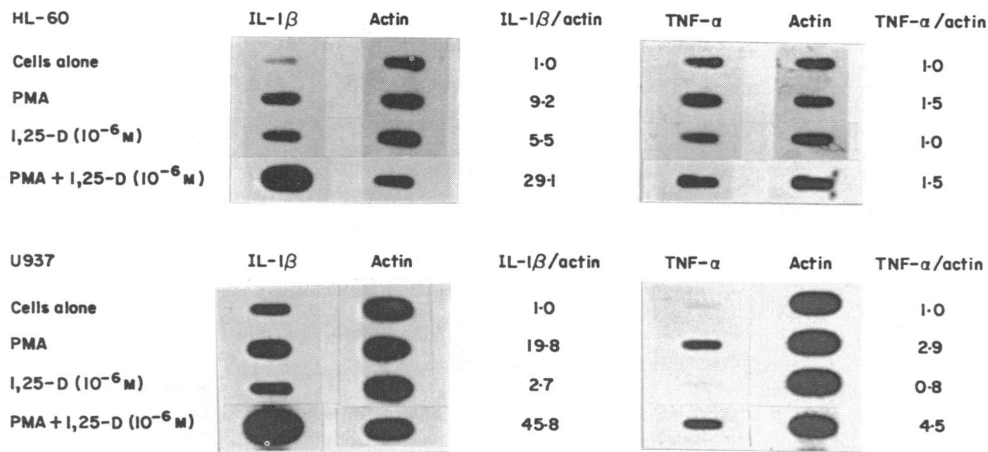

Figure 2. Slot blot analysis of IL-1β and TNF-α mRNA expression in HL-60 and U937 cells treated with PMA, 1,25-$(OH)_2D_3$ or both. The same filters were also hybridized with chicken β-actin to determine the amount of RNA loaded. Relative slot blot densities are shown for cytokine/β-actin mRNA normalized as 1·0 for the ratio of densities obtained with mRNA from unstimulated cells.

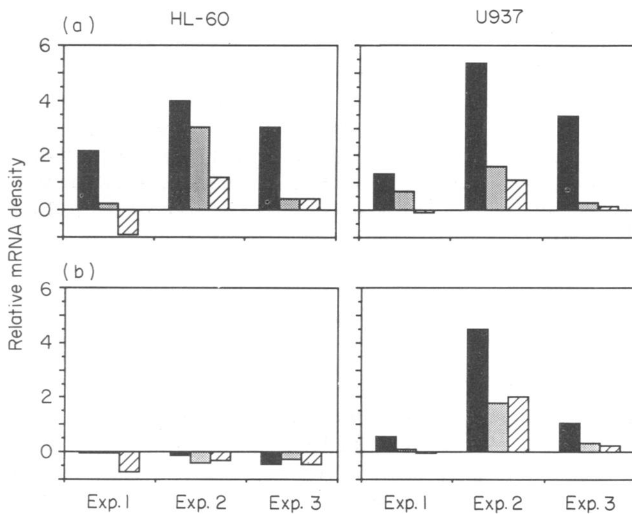

Figure 3. The effect of 1,25-$(OH)_2D_3$ on IL-1β (a) and TNF-α (b) mRNA expression in PMA-stimulated HL-60 and U937 cells. Three separate experiments (slot blotting). Horizontal line at 0 represents the relative mRNA density (cytokine/β-actin) in PMA-stimulated cultures without 1,25-$(OH)_2D_3$. Black columns: 10^{-6} M 1,25-$(OH)_2D_3$. Shaded columns; 10^{-8} M 1,25-$(OH)_2D_3$. Striped columns: 10^{-10} M 1,25-$(OH)_2D_3$.

lineage[17] and respond differently to retinoic acid.[10,18] In these studies it was demonstrated that retinoic acid had anti-proliferative effects on U937 cells but it did not induce differentiation in these cells. In HL-60 cells retinoic acid also has anti-proliferative effects but does induce differentiation of these cells towards granulocytes.[18] Both PMA and 1,25-$(OH)_2D_3$ cause the differentiation of HL-60 and U937 cells towards more mature monocyte/macrophages. However, the differentiation steps induced in these cell lines by PMA or by 1,25-$(OH)_2D_3$ must differ as only PMA is capable of inducing the TNF-α gene in the particular cell lines which we studied.

A recent report suggested that 1,25-$(OH)_2D_3$ alone was capable of increasing the transcription of the TNF-α gene in HL-60 cells.[12] We were unable to confirm this as the hormone failed to cause TNF-α mRNA expression or to augment that which had been induced by PMA. An explanation for this discrepancy could relate to the varied behaviour of different sublines of HL-60 as has been observed with sublines of U937 cells. Some lines of U937 respond to LPS with respect to transcription of the IL-1 gene, while others remain unresponsive[16,19] and these differences may be related to the mycoplasma contamination often seen in cell lines including U937.[20] Both the U937 and the HL-60 cell lines used in our own study were shown not to be contaminated with mycoplasma. An alternative explanation for the observed differences might be genetic divergence in different sublines of HL-60.

1,25-$(OH)_2D_3$ can induce the differentiation of myeloid leukaemia cell lines to more mature monocytic cells. 1,25-$(OH)_2D_3$ may also serve to activate already differentiated monocytes/macrophages. The hormone has been shown to inhibit the growth of *Mycobacterium tuberculosis* inside the macrophage[21] and to enhance the production of oxygen metabolites by human monocytes during the respiratory burst.[11] In addition, human monocytes and macrophages produce the enzyme 25-OH D_3-1-hydroxylase, which converts inactive 25-hydroxyvitamin D_3 to the biologically active metabolite 1,25-$(OH)_2D_3$. Our findings demonstrate that 1,25-$(OH)_2D_3$ can differentially modify the regulation of IL-1β and TNF-α genes in different myeloid cell lines. The role of mRNA stability and translational control in the production of these cytokines was not addressed in the present study, and therefore we are unable to directly relate the observed increases in cytokine mRNA levels to previous observations on secretion of IL-1β and TNF-α by myeloid cells. However, the present findings lend support to previous observations suggesting that the hormone can regulate cytokine production by mature peripheral blood monocytes.[4] Taken together the above findings suggest that 1,25-$(OH)_2D_3$ may play an important role at local sites of inflammation by exerting a paracrine or autocrine action on monocyte differentiation and on macrophage activation and function.

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