Redox regulation of cAMP levels by ascorbate in 1,25-dihydroxy-vitamin D₃-induced differentiation of HL-60 cells

Guillermo LÓPEZ-LLUCH*, M. Isabel BURÓN*, Francisco J. ALCAÍN*, Jose M. QUESADA† and Plácido NAVAS*¹

*Departamento de Biología Celular, Facultad de Ciencias, Universidad de Córdoba, 14004 Córdoba, Spain, and †Unidad de Metabolismo Mineral, Hospital Reina Sofía, 14004 Córdoba, Spain

1 α ,25-Dihydroxyvitamin D₃ [1,25-(OH)₂D₃] induces differentiation to monocyte-macrophage lineage of several leukaemic cell lines such as HL-60, U937, M1 and Mono Mac 6. Ascorbate also modulates growth and differentiation of different animal cells in culture. We have previously reported the stimulating effect of ascorbate on 1,25-(OH)₂D₃-induced HL-60 cell differentiation. We show here that 1,25-(OH)₂D₃ induces a transient increase in cAMP levels in these cells, and ascorbate significantly increases these cAMP levels. Ascorbate alone does not have any effect. Other cAMP-increasing agents such as isobutylmethylxanthine, forskolin and prostaglandin E₂ maintain high levels of cAMP at 48 h of incubation and also enhance differentiation along the monocytic pathway induced by 1,25-(OH)₂D₃, as revealed by specific differentiation markers, demonstrating the importance of cAMP in the differentiation process. It is also shown that the presence of ascorbate and its free radical (AFR) during 1,25-(OH)₂D₃-induced differentiation significantly de-

creases cytoplasmic NADH levels compared with those induced by 1,25-(OH)₂D₃ in HL-60 cells. The results indicate that NADH is an inhibitor of adenylate cyclase in these cells. AFR is an electron acceptor of the trans-plasma-membrane electron-transport system, and NADH is the electron donor. Through this system, ascorbate and AFR keep levels of NADH low, thereby decreasing its inhibitory effect on adenylate cyclase activity and so increasing cAMP synthesis. We also demonstrate that other ascorbate derivatives, such as ascorbate 2-phosphate and dehydroascorbate, both of which are unable to produce AFR, do not alter intracellular NADH levels during 1,25-(OH)₂D₃-induced differentiation. Also, ascorbate and AFR increase specific differentiation markers (CD14 and NitroBlue Tetrazolium reduction) but neither ascorbate 2-phosphate nor dehydroascorbate show this enhancing activity. In summary, we propose that the effect of ascorbate on 1,25-(OH)₂D₃-induced differentiation of HL-60 cells can be explained by redox regulation of the cAMP pathway.

INTRODUCTION

The hormonally active metabolite of the vitamin D endocrine system, 1 α ,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃), plays an important regulatory role in different cell functions, such as proliferation and differentiation in both normal and cancerous cells [1,2]. Since Suda and co-workers [3] reported the 1,25-(OH)₂D₃ induction of differentiation of a mouse myeloid leukaemic cell line, many other groups have demonstrated its potential in growth and differentiation of both normal and leukaemic cell lines to monocyte-macrophage phenotype [4–6]. Further, 1,25-(OH)₂D₃ also acts on the differentiation of solid tumours *in vitro* [7] and *in vivo* [8], suggesting its potential therapeutic use for human malignancies.

Ascorbate is a water-soluble vitamin classically known as the active factor in the hydroxylation of collagen and the formation of its functional triple helix [9], and is involved in many other physiological functions [10]. It is also involved in the modulation of cell growth and differentiation in non-collagen-related cell lines, such as leukaemic cells [11,12]. It has been observed that ascorbate exerts its function by stimulation of the hapten stimulus [13]. Thus ascorbate does not induce cell differentiation itself, but stimulates 1,25-(OH)₂D₃-induced differentiation in osteosarcoma [14] and HL-60 cells [15]. In a previous report [15], we showed stimulation by ascorbate of the differentiation of the myelocytic cells (HL-60) induced by 1,25-(OH)₂D₃, demonstrated by an increase in the appearance of differentiation markers such as

CD11b (a monocyte- and granulocyte-specific antigen corresponding to the α -subunit of complement factor 3 receptor) and CD14 (a monocyte-macrophage receptor specific for bacterial lipopolysaccharide) cell-surface antigens and cytochemical markers such as NitroBlue Tetrazolium (NBT) reduction and non-specific esterase (NSE) activity.

Ascorbate is quickly oxidized to dehydroascorbate (DHA), and the first product of its oxidation, the ascorbate free radical (AFR), acts mainly as an oxidant in biological systems [16]. Eukaryotic plasma membrane contains a transmembrane electron-transport system involved in the regulation of growth and development [17]. NADH-AFR reductase is one component of this redox system in HL-60 cells which, by maintaining ascorbate in its reduced state at the cell surface [18], regulates cytosolic NADH/NAD⁺ levels [12,19] and as a consequence participates in the regulation of the cytosolic redox state.

The involvement of cAMP in a haematopoiesis and granulocytic differentiation has been observed [20–22]. We have also produced preliminary data for a role for cAMP in monocytic differentiation of HL-60 cells induced by 1,25-(OH)₂D₃ [15]. We report here that the addition of several cAMP-increasing agents enhanced the monocytic differentiation induced by 1,25-(OH)₂D₃, suggesting that the cAMP pathway is an important mechanism in the mediation of the differentiation induced by 1,25-(OH)₂D₃ in HL-60 cells. Ascorbate potentiates the differentiation by increasing cAMP only in the presence of 1,25-(OH)₂D₃. The ascorbate effect is mediated by its free radical, which decreases

Abbreviations used: 1,25-(OH)₂D₃; 1 α ,25-dihydroxyvitamin D₃, calcitriol; AFR, ascorbate free radical; Asc-2-P, ascorbate 2-phosphate; DHA, dehydroascorbate; IBMX, isobutylmethylxanthine, MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; NBT, NitroBlue Tetrazolium; NSE, non-specific esterase; PGE₂, prostaglandin E₂; MFI, mean fluorescence intensity.

¹ To whom correspondence should be addressed.

NADH concentrations induced by 1,25-(OH)₂D₃. Since NADH inhibits adenylate cyclase, the lower levels of NADH caused by AFR favour the production of cAMP and consequently differentiation. Thus our results support the idea that 1,25-(OH)₂D₃-induced differentiation of HL-60 cells is regulated by redox mechanisms mediated by ascorbate.

MATERIALS AND METHODS

Cell culture

HL-60 cells were cultured in RPMI 1640 medium (Sigma, Madrid, Spain) supplemented with 10% heat-inactivated fetal calf serum (PAA Labor), 100 units/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml amphotericin B (Sigma), and maintained at 37 °C in a humidified atmosphere of 5% CO₂ in air. HL-60 cells (10⁵/ml) were cultured in multiwell plates and induced to differentiate with 1,25(OH)₂D₃ (kindly provided by Solvay-Duphar) dissolved in ethanol as vehicle. As the final concentration of ethanol in the culture medium was 0.1%, and it had no effect on either cell growth or differentiation, the same volume of ethanol was added to control cultures.

Treatments

Isobutylmethylxanthine (IBMX), DMSO, prostaglandin E₂ (PGE₂) and forskolin were obtained from Sigma. IBMX was dissolved in sterile 0.1 M NaOH at a concentration 1000-fold higher than that achieved in cell culture, forskolin was dissolved in DMSO in a 5 mM stock solution, and PGE₂ was dissolved in ethanol in a 10 mM stock concentration. Cultures containing 0.2% DMSO were used as a control of forskolin addition. Ascorbate (sodium salt; Aldrich), DHA (Sigma) and ascorbate 2-phosphate (Asc-2-P) (Wako Pure Chemical Industries) were dissolved in sterile distilled water at a 20 mM stock concentration and added at the beginning and after every 48 h of culture because of its oxidation. Every 48 h, two-thirds of the culture medium was removed and replaced with fresh medium containing 1,25(OH)₂D₃, IBMX, PGE₂, DMSO, forskolin, ascorbate, DHA or Asc-2-P to maintain initial concentrations. Viability was over 90% during the treatments as determined by the Trypan Blue-exclusion method. During the treatments, a few cells became adherent but, in each case, they were detached with a 'rubber policeman' and analysed together with the non-adherent cells.

Cytochemical assays

NBT reduction was determined cytochemically as described by Yun and Sugihara [23]. Cells were counterstained with Eosin G and the percentage of positive cells showing blue formazan deposits was determined by counting under a light microscope. NSE activity was determined as described previously [24]. In both cases, at least 300 cells were counted for each treatment.

Determination of surface antigen

The presence of the surface antigens CD11b and CD14 was determined by flow immunocytometry. CD11b corresponds to the C3bi complement receptor and was detected by direct immunofluorescence using a FITC-labelled monoclonal antibody (Sigma). CD14 expression was determined by direct immunofluorescence using a phycoerythrin-labelled monoclonal antibody (Sigma). Cells were previously washed with PBS and then incubated with the respective monoclonal antibody at 4 °C for 30 min, washed twice, fixed in 1% formalin in PBS and analysed. Negative controls were treated with mouse isotype controls that were FCIT or phycoerythrin labelled. In each determination, at

least 5000 cells were collected by a FACsort and analysed by LysisII software (Becton-Dickinson).

Assay of cAMP

Cytoplasmic cAMP levels were determined using the Amersham [³H]cAMP assay kit (Amersham, Iberica, Spain). Cells were removed and quickly washed in ice-cold 100 mM Tris-HCl, pH 7.4. They were then lysed by incubation with 4 mM EDTA and a brief sonication, and an aliquot was collected for protein determination. Afterwards, samples were deproteinated by heating in boiling water for 10 min and protein was removed by centrifugation at 14000 g (12000 rev./min) in a Microfuge for 2 min. cAMP levels in the supernatant were determined as described in the assay kit protocol using a liquid-scintillation counter LS600TA (Beckman). Protein was determined by the method of Bradford [25].

Assay of adenylate cyclase

Adenylate cyclase activity was determined as described [26]. In brief, HL-60 crude membranes (50 µg of protein) were incubated at 32 °C for 20 min in a final volume of 155 µl containing 20 mM Hepes, pH 7.8, 10 mM phosphocreatine, 0.2 mg/ml creatine kinase, 2.5 mM MgCl₂, 100 µM IBMX, 50 µM GTP and 100 µM ATP (Sigma). Reaction was stopped by the addition of 100 µl of methanol/chloroform (1:1, v/v), and the solution was mixed and centrifuged at 14000 g in a Microfuge for 5 min, and 50 µl of the cAMP-containing aqueous phase was measured using the specific [³H]cAMP assay kit (Amersham).

Determination of nicotinamide nucleotides

NADH was extracted by the method of Jacobson and Jacobson [27]. Briefly, cells were washed in ice-cold PBS and 10⁶ cells were resuspended in 1 ml of PBS. NADH was extracted by alkaline lysis with 1 ml of 0.25 M NaOH. After 5 min at room temperature, lysate was centrifuged for 10 min at 1500 g and, finally, supernatant was neutralized with 0.33 M phosphate buffer, pH 7.5, and 0.37 M phosphoric acid. Extracts were frozen at -80 °C until determination.

NADH was determined by the cyclic assay as described previously [28]. Extracts were mixed with the reaction mixture [final concentrations 0.5 M ethanol, 4 mM EDTA, 1.66 mM phenazine ethosulphate, 0.2 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) in 100 mM Bicine/NaOH buffer, pH 8] and at a final volume of 1 ml (EDTA, phenazine ethosulphate, MTT and Bicine from Sigma). The reaction was started by the addition of 20 µl of 500 U/ml alcohol dehydrogenase (EC 1.1.1.1; Sigma), and incubation was carried out at 37 °C for 30 min. MTT reduction was measured spectrophotometrically at 570 nm, and nicotinamide nucleotides were quantified in accordance with a standard curve obtained under the same conditions with known concentrations of reduced nucleotides.

Determination of arachidonic acid release from HL-60 cells

HL-60 cells (1 × 10⁶/ml) were labelled with 1 µCi/well [³H]arachidonic acid (Amersham) for the last 12 h of the incubation at 37 °C. After labelling, the medium was removed by centrifugation at 1000 g for 5 min, and the pellet washed twice with 10 ml of serum-free RPMI 1640 medium. Labelled cells were resuspended in 1 ml of RPMI 1640 plus 10% fetal calf serum with or without the respective treatment. After 1 h of incubation, medium was collected by centrifugation and radioactivity was measured using a liquid-scintillation counter.

Statistical analysis

Data represent means $\pm$ (S.D.) from at least three different experiments performed in triplicate for cytochemical assays or in duplicate for surface antigens, cAMP determination, arachidonic acid release and nicotinamide nucleotide assay. Significant differences between different treatments were analysed using Student's *t* test.

RESULTS

Incubation of HL-60 cells with 10⁻⁸ M 1,25-(OH)₂D₃ induces an increase in cAMP after 48–72 h [15]. The effect of 1,25-(OH)₂D₃ was dependent on concentration, and cAMP levels reached a plateau with 10⁻⁷ M 1,25-(OH)₂D₃ (Figure 1). The concentrations

Figure 1 cAMP levels in HL-60 cells with different concentrations of 1,25-(OH)₂D₃

HL-60 cells, plated at 10⁵/ml, were treated with the indicated concentrations of 1,25-(OH)₂D₃ dissolved in ethanol. Control cultures contained 0.1% ethanol. Cells were removed after 48 h of treatment, and cAMP levels determined as described in the Materials and methods section. Data represent means $\pm$ S.D. from three different experiments performed in duplicate. * *P* $\leq$ 0.01 compared with control.

Table 1 Effect of cAMP-increasing agents and ascorbate on cAMP levels in 1,25-(OH)₂D₃-treated HL-60 cells

Cells were plated at 10⁵ cells/ml and treated, in the presence or absence of 10⁻⁸ M 1,25-(OH)₂D₃, with 1 mM IBMX, 10 μ M forskolin, 10 μ M PGE₂, 0.2% DMSO or 0.2 mM ascorbate. Cells were removed after 48 h, and cAMP levels were determined. Data represent means $\pm$ S.D. from three different experiments performed in duplicate. * *P* $\leq$ 0.05 compared with un-treated cells; † *P* $\leq$ 0.05 compared with 1,25-(OH)₂D₃ treatment alone.

Treatment	cAMP (pmol)	
	Control	1,25-(OH) ₂ D ₃
None	31.06 $\pm$ 11.48	122.19 $\pm$ 12.18
IBMX (1 mM)	132.55 $\pm$ 26.00*	282.60 $\pm$ 80.31†
Forskolin (10 μ M)	66.16 $\pm$ 5.96*	360.46 $\pm$ 72.35†
DMSO (0.2%)	38.38 $\pm$ 9.11	113.64 $\pm$ 18.79
PGE ₂ (10 μ M)	32.33 $\pm$ 1.12	253.62 $\pm$ 26.45†
Ascorbate (0.2 mM)	34.71 $\pm$ 11.41	222.11 $\pm$ 30.56†

Table 2 Effect of cAMP-increasing agents and ascorbate on CD11b and CD14 expression in 1,25-(OH)₂D₃-differentiated HL-60 cells

Cells were incubated as described in Table 1 and removed after 48 h. The percentage of CD11b- and CD14-positive cells was determined by flow cytometry as described in the Materials and methods section. At least 5000 cells were counted for each sample. Data represent means $\pm$ S.D. from three different experiments performed in duplicate. * *P* $\leq$ 0.05 compared with untreated cells; † *P* $\leq$ 0.05 compared with 1,25-(OH)₂D₃ treatment alone.

Treatment	CD11b (% positive cells)		CD14 (% positive cells)	
	Control	1,25-(OH) ₂ D ₃	Control	1,25-(OH) ₂ D ₃
None	3.06 $\pm$ 1.19	27.44 $\pm$ 8.35	3.02 $\pm$ 0.92	51.06 $\pm$ 9.30
IBMX (1 mM)	14.66 $\pm$ 5.98*	34.80 $\pm$ 4.99†	6.65 $\pm$ 2.38	64.80 $\pm$ 5.80†
Forskolin (10 μ M)	10.12 $\pm$ 3.08*	92.55 $\pm$ 0.54†	4.30 $\pm$ 2.03	97.13 $\pm$ 0.98†
DMSO (0.2%)	9.15 $\pm$ 5.03	28.52 $\pm$ 9.89†	2.09 $\pm$ 1.92	52.21 $\pm$ 3.44
PGE ₂ (10 μ M)	3.27 $\pm$ 1.31	80.49 $\pm$ 4.95†	2.72 $\pm$ 1.01	87.10 $\pm$ 5.56†
Ascorbate (0.2 mM)	7.92 $\pm$ 2.85	34.81 $\pm$ 4.99	3.52 $\pm$ 1.07	73.40 $\pm$ 7.85†

of 1,25-(OH)₂D₃ that produced a significant increase in cAMP in HL-60 cells (Figure 1) were observed to produce significant differentiation in these cells [15].

Agents able to increase cAMP were used to verify the role of cAMP in the differentiation programme. HL-60 cells were treated with 10⁻⁸ M 1,25-(OH)₂D₃ in the presence or absence of the cAMP-increasing agents, forskolin, IBMX and PGE₂, as well as ascorbate, and cAMP levels were determined after 48 h (Table 1). Only 1 mM IBMX and 10 μ M forskolin induced significant increases in cAMP levels in both non-induced and 1,25-(OH)₂D₃-induced cells. Forskolin was more efficient, inducing a 3-fold increase in the cAMP concentration in 1,25-(OH)₂D₃-treated cells, but its effect on non-induced cells was only 80% of increase. The effect of IBMX appeared to be additive because it increased cAMP levels significantly by itself in both non-induced and induced cells. Both 10 μ M PGE₂ and 0.2 mM ascorbate only caused a 2-fold cAMP increase in 1,25-(OH)₂D₃-induced cells, but had no effect in non-induced cells. DMSO, used as the vehicle for forskolin, did not have any effect on cAMP levels.

Cell-surface markers for HL-60 cell monocytic differentiation, CD11b and CD14, were analysed at 48 h of incubation, with the same agents and conditions as in Table 1; the results are given in Table 2. Both forskolin and PGE₂ induced a significant increase in the number of positive cells for these two surface antigens

Table 3 CD11b and CD14 MFI in HL-60 cells treated with 10⁻⁸ M 1,25-(OH)₂D₃ and different cAMP-increasing agents

Cells were treated for 48 h with ethanol (control) or 10⁻⁸ M 1,25-(OH)₂D₃ with the addition of 0.2 mM ascorbate, 10 μ M forskolin, 1 mM IBMX, 10 μ M PGE₂ or 0.2% DMSO. Data represent the MFI of treatments indicated in Table 2. * *P* $\leq$ 0.05 compared with 1,25-(OH)₂D₃ treatment alone.

Treatments	CD11b		CD14	
	Control	1,25-(OH) ₂ D ₃	Control	1,25-(OH) ₂ D ₃
None	31.22	36.52	36.30	125.34
IBMX (1 mM)	25.00	52.53*	32.07	58.62*
Forskolin (10 μ M)	32.75	49.36*	28.00	251.11*
DMSO (0.2%)	30.50	35.63	29.32	130.03
PGE ₂ (10 μ M)	29.59	34.42	32.10	155.03*
Ascorbate (0.2 mM)	29.70	35.50	27.80	142.58*

Table 4 Effect of cAMP-increasing agents and ascorbate on the appearance of NBT reducing activity in 1,25-(OH)₂D₃-differentiated HL-60 cells

Cells were incubated as described in Table 3 and removed after 72 h of treatment. The percentage of NBT-positive cells was determined by cytochemistry as described in the Materials and methods section. At least 300 cells were counted for each sample. Data represent means $\pm$ S.D. from three different experiments performed in triplicate. * $P \leq 0.05$ compared with 1,25-(OH)₂D₃ treatment alone.

Treatment	NBT-positive cells (%)	
	Control	1,25-(OH) ₂ D ₃
None	4.91 $\pm$ 2.23	11.85 $\pm$ 1.18
IBMX (1 mM)	7.05 $\pm$ 1.19	25.45 $\pm$ 4.39*
Forskolin (10 μ M)	5.83 $\pm$ 1.90	47.83 $\pm$ 2.25*
DMSO (0.2%)	6.76 $\pm$ 0.43	15.01 $\pm$ 1.85
Ascorbate (0.2 mM)	5.00 $\pm$ 2.17	18.29 $\pm$ 2.12*

when they were added as a supplement to 1,25-(OH)₂D₃ treatments. In both cases, the enhancing effect of forskolin was the highest, reaching nearly 100 % of positive cells after this length of incubation. The vehicle for forskolin, DMSO, did not produce any effect on surface antigen expression in either non-induced or 1,25-(OH)₂D₃-treated cells, although a small but not significant effect was observed with DMSO itself. Also, IBMX and ascorbate only had an increasing effect on the percentage of CD14-positive cells and not CD11b-positive cells. Further, only IBMX and forskolin, agents that increased cAMP concentration by themselves in non-induced cells, had a slight but significant effect on CD11b expression in cultures without 1,25-(OH)₂D₃.

The presence of both cell-surface antigens on positive cells was also increased, as determined by mean fluorescence intensity (MFI). Forskolin, PGE₂ and ascorbate increased CD14 MFI when added to 1,25-(OH)₂D₃-treated cells, but IBMX, which was shown to increase the percentage of CD14-positive cells (Table 2), caused a decrease in this antigen in 1,25-(OH)₂D₃-treated cells (Table 3). In the case of CD11b, only forskolin and IBMX increased the MFI when added to 1,25-(OH)₂D₃-treated cells. DMSO did not have any effect, and, for all treatments no effect was observed on the MFI of non-induced cells (Table 3).

The acquisition of the functional phenotype of mature monocytes was determined by the presence of NBT-reducing cells. Cells growing in the presence or absence of 1,25-(OH)₂D₃ were treated with the most effective cAMP-increasing agents, i.e. forskolin and IBMX, and compared with ascorbate (Table 4). Although changes in cAMP concentrations were observed after 48 h, NBT-positive cells do not appear until after this length of time [15], so we determined the presence of NBT-positive cells after 72 h of treatment. The three agents tested here increased the percentage of 1,25-(OH)₂D₃-induced NBT-positive cells, but not the non-induced cells. DMSO did not have any effect on either (Table 4).

Also, we determined cytochemically the presence of NSE activity, an activity that appears later in the maturation process of monocytes. In this case, we used forskolin which was the most effective agent in both enhancement of cAMP and differentiation in 1,25-(OH)₂D₃-treated cells. In comparison with the effect of ascorbate, forskolin was more effective when added with 10⁻⁸ M 1,25-(OH)₂D₃, but showed the same potentiating effect as ascorbate when added with 10⁻⁷ M 1,25-(OH)₂D₃ (Table 5). Neither agents produced any effect on non-induced cells and DMSO did not have any effect on either non-induced or 1,25-(OH)₂D₃-treated cells (results not shown).

Table 5 Effect of forskolin and ascorbate on the presence of NSE activity in 1,25-(OH)₂D₃-differentiated HL-60 cells

Cells were incubated as described in Table 1 and removed after 96 h of treatment. The percentage of NSE-positive cells was determined cytochemically as described in the Materials and methods section. At least 300 cells were counted for each sample. Data represent means $\pm$ S.D. from three different experiments performed in triplicate. * $P \leq 0.05$ compared with 1,25-(OH)₂D₃ treatment alone.

Treatment	NSE-positive cells (%)		
	Control	10 ⁻⁸ M 1,25-(OH) ₂ D ₃	10 ⁻⁷ 1,25-(OH) ₂ D ₃
None	3.25 $\pm$ 1.30	11.98 $\pm$ 2.50	40.32 $\pm$ 5.85
Ascorbate (0.2 mM)	2.66 $\pm$ 2.03	37.91 $\pm$ 2.61*	61.20 $\pm$ 7.34*
Forskolin (10 μ M)	2.90 $\pm$ 0.78	52.80 $\pm$ 3.78*	61.99 $\pm$ 2.74*

Mechanisms involved in the production of cAMP by forskolin, IBMX or PGE₂ have been characterized, but no information is available about the action of ascorbate on this phenomenon. A possible mechanism of ascorbate stimulation of 1,25-(OH)₂D₃-induced differentiation of HL-60 cells is based on enhanced release of arachidonic acid metabolites, such as PGE₂. Therefore the release of arachidonic acid from HL-60 cells treated with 1,25-(OH)₂D₃ was studied in the presence of ascorbate. Cells were incubated for 48 h with several concentrations of 1,25-(OH)₂D₃ (10⁻⁹ M–10⁻⁷ M), and the release of arachidonic acid-derived metabolites was determined. 1,25-(OH)₂D₃ induced an increase in the release of arachidonic acid metabolites, but the addition of ascorbate had no effect (Table 6).

Another possible mechanism of the action of ascorbate on 1,25-(OH)₂D₃-induced cells is based on ascorbate metabolism. Thus 1,25-(OH)₂D₃-induced cells were incubated with different forms of ascorbate, and CD14- and NBT-positive cells were determined (Table 7). Both ascorbate and its free radical, AFR, had the same effect on these two markers of HL-60 cell differentiation. Neither Asc-2-P, a non-oxidizable form, nor DHA, the fully oxidized form, had any effect on 1,25-(OH)₂D₃-induced differentiation of HL-60 cells.

As ascorbate and its free radical induce changes in the cellular

Table 6 Effect of ascorbate on the release of [³H]arachidonic acid induced by 1,25-(OH)₂D₃ in HL-60 cells

Cells were treated for 48 h with the indicated concentrations of 1,25-(OH)₂D₃ in the presence or absence of 0.2 mM ascorbate. They were loaded with [³H]arachidonic acid for the last 12 h of the incubation and metabolite release was determined as described in the Materials and methods section. Data represent means $\pm$ S.D. from three different experiments performed in duplicate. * $P \leq 0.05$ compared with control (no treatment).

Treatment	Ascorbate addition	[³ H]Arachidonic acid release (d.p.m./h per mg of protein)
None	—	15 050 $\pm$ 2885
	+	18 745 $\pm$ 2359
10 ⁻⁹ M 1,25-(OH) ₂ D ₃	—	17 019 $\pm$ 2548
	+	18 420 $\pm$ 2556
10 ⁻⁸ M 1,25-(OH) ₂ D ₃	—	22 950 $\pm$ 3190*
	+	23 960 $\pm$ 3260
10 ⁻⁷ M 1,25-(OH) ₂ D ₃	—	23 554 $\pm$ 1249*
	+	23 570 $\pm$ 2050

Table 7 Effects of ascorbate redox forms on HL-60 cells differentiation induced by 1,25-(OH)₂D₃ and the NADH content

Cells treated with 10⁻⁸ M 1,25-(OH)₂D₃ in the presence or absence of different ascorbate redox forms at a concentration of 0.2 mM were incubated for 48 h for the NADH and CD14 determinations and for 72 h for the assay of NBT reduction. Data represent means ± S.D. from three different experiments performed in duplicate. * *P* ≤ 0.05 compared with control (no treatment).

Treatment	CD14-positive cells (%)	NBT-positive cells (%)	NADH content (pmol/mg of protein)
None	50.1 ± 9.0	11.5 ± 1.2	700 ± 22.6
Ascorbate	74.8 ± 7.8*	22.5 ± 3.3*	580 ± 18.5*
AFR	80.3 ± 4.2*	23.4 ± 2.3*	550 ± 20.6*
Asc-2-P	52.6 ± 6.3	10.5 ± 2.5	680 ± 15.4
DHA	47.8 ± 4.3	13.1 ± 3.6	730 ± 26.2

Figure 2 NADH levels during HL-60 cell differentiation induced by 1,25-(OH)₂D₃ in the presence or absence of ascorbate

Cells were collected after the indicated times of incubation, lysed and NADH levels in the cell extracts were determined as described in the Materials and methods section. Data represent mean ± S.D. from three different experiments performed in duplicate. * *P* ≤ 0.01 compared with respective control or 1,25-(OH)₂D₃ treatments without ascorbate.

concentration of NADH [19], we measured NADH in HL-60 cells treated as described above. Table 7 shows a correlation between the decrease in NADH and the increase in CD14- and NBT-positive cells in 1,25-(OH)₂D₃-induced cells treated with both ascorbate and AFR, but no changes in NADH concentration were observed in the presence of Asc-2-P or DHA.

The time course of the effect of ascorbate on NADH concentration in HL-60 cells induced by 1,25-(OH)₂D₃ was analysed to determine its role as a redox compound (Figure 2). The induction to differentiation with 1,25-(OH)₂D₃ increased the concentration of NADH over the first 60 h; NADH levels then fell, reaching basal levels at 72 h. The presence of ascorbate significantly decreased these concentrations throughout the treatment, and at 72 h the levels were similar to initial levels. NADH concentrations of non-induced cells remained constant through-

Figure 3 Effect of NADH on adenylate cyclase activity of HL-60 cell membranes

Crude HL-60 membranes were incubated with increasing concentrations of NADH, and adenylate cyclase activity was assayed over 20 min. Adenylate cyclase activity and cAMP were determined as described in the Materials and methods section. Data represent means ± S.D. from three different experiments performed in duplicate. * *P* ≤ 0.01 compared with control (no NADH).

out the experiment. Ascorbate itself induced a slight but significant decrease at 24 h relative to control cells.

As NADH clearly appears to be involved in the differentiation pathway induced by 1,25-(OH)₂D₃ and has been shown to inhibit adenylate cyclase [29,30], we determined the effect of NADH on the adenylate cyclase activity of HL-60 cells. Adenylate cyclase activity was strongly inhibited by NADH, being almost completely abolished at 5 mM (Figure 3).

DISCUSSION

1,25(OH)₂D₃-induced differentiation has been described in many target tissues, the most complex events occurring in haematopoietic cells [31]. The HL-60 cell line has been a classic target for *in vitro* studies of induced differentiation, and it has been known since 1983 that 1,25-(OH)₂D₃ induces its differentiation to monocytes [4]. Different mechanisms have been associated with the differentiation induced by 1,25-(OH)₂D₃ and have also been described using other differentiating agents such as PMA, retinoic acid and DMSO [31]. Differentiation of HL-60 cells to granulocytes can be achieved by activators of adenylate cyclase such as prostaglandins and adrenaline [32,33], or the permeable analogue dibutyryl cAMP [21]. Also, retinoic acid reverses the malignant phenotype of HL-60 cells through the activation of the cAMP-dependent protein kinase [34].

1,25(OH)₂D₃ elevates cAMP levels in classic tissue targets such as myoblasts, osteogenic sarcoma cells, skeletal muscle, T47D human breast cancer cells, human melanocytes and intestinal cells [31,35]. We have previously shown a transient increase in cytosolic cAMP in HL-60 cells induced by 1,25-(OH)₂D₃ [15]. We show here that this increase is dose-dependent and reaches the highest levels above 10⁻⁷ M 1,25(OH)₂D₃. The expression of monocyte differentiation markers induced by different concentrations of 1,25-(OH)₂D₃ [15] correlated with the concentration of cytosolic cAMP observed here. In agreement with these results, co-operation of dibutyryl cAMP with 1,25-(OH)₂D₃ to

induce HL-60 cell differentiation has been reported [36]. Also, a decrease in cytosolic cAMP in HL-60 cells treated with uraemic serum correlates with resistance to 1,25-(OH)₂D₃-induced differentiation [37]. Further, the effect of different cAMP-increasing agents on differentiation shown here clearly involves cAMP. It seems that the differentiation of HL-60 cells depends specifically on the protein kinase A activation pathway in both commitment and mature-phenotype-expression phases [38]. Ascorbate also stimulates both the increase in cAMP levels and the appearance of differentiation markers in HL-60 cells induced by 1,25-(OH)₂D₃. Thus the potentiating effect of ascorbate on 1,25-(OH)₂D₃-induced differentiation of leukaemic cells must involve the production of cAMP.

The mechanism by which 1,25(OH)₂D₃ increase cAMP levels in HL-60 cells remains unknown. We have found that PGE₂, which is synthesized by HL-60 cells in response to 1,25(OH)₂D₃, elevates cAMP and stimulates 1,25(OH)₂D₃-induced differentiation of these cells. However, inhibition of PGE₂ synthesis with indomethacin and aspirin does not prevent 1,25(OH)₂D₃-induced differentiation of HL-60 cells but actually increases the differentiation process [32]. Also, here we have shown that, although 1,25-(OH)₂D₃ induced the release of arachidonic acid-related compounds in HL-60 cells, the presence of ascorbate did not change this activity. We can therefore assume that the increase in cAMP induced by 1,25(OH)₂D₃ and increased by ascorbate is not due to an autocrine mechanism of PGE₂. It has been shown that 1,25(OH)₂D₃ increases adenylate cyclase activity in muscle cells through the phosphorylation of G_iα-protein [35,39]. Thus the modulation of adenylate cyclase activity appears to be an early event in the induction of differentiation by 1,25-(OH)₂D₃, and the increase in cAMP production by ascorbate, like to those agents that stimulate this activity, must be the mechanism of its stimulating role in differentiation.

Ascorbate does not modify the phenotype of HL-60 cells but stimulates specific differentiation to the monocyte phenotype induced by 1,25-(OH)₂D₃ [15]. This is in agreement with the idea that ascorbate only acts in properly induced cells [13,40]. Ascorbate is involved in many physiological functions [9] and modulates growth and differentiation of several mesenchyme-derived cells lines [11,41]. Although ascorbate appears to stimulate cell differentiation by inducing the stable collagen-matrix-associated signal [9], we have demonstrated ascorbate participation in the differentiation programme induced by 1,25-(OH)₂D₃ on monocyte-macrophage progenitor cells through the cAMP pathway, and thus is a new role for ascorbate in cell metabolism.

Ascorbate is a redox compound that undergoes a two-step oxidation in aqueous solution. The first product of this oxidation, AFR, acts mainly as an oxidant [16]. Thus ascorbate can act as either an antioxidant [42,43] or as an oxidant via AFR [13,16]. This free radical potentiates growth in serum-limiting medium [12], and also stimulates NF-κB induced by tumour necrosis factor α, decreasing growth in T-cells [40]. AFR, but not Asc-2-P or DHA, produced similar stimulating effects to those of ascorbate in the 1,25-(OH)₂D₃-induced differentiation of HL-60 cells. Ascorbate apparently acts as an oxidant via AFR in the stimulation observed in this work. AFR is an extracellular acceptor in the trans-plasma-membrane electron-transport system [44], and its action on this system induces a decrease in cellular NADH content [12,19]. 1,25-(OH)₂D₃ produced an increase in NADH during the induction of HL-60-cell differentiation. Also, the NADH/NAD⁺ ratio is increased in PMA-induced differentiation of HL-60 cells [45,46]. However, the presence of ascorbate or its free radical prevented the increase in NADH induced by 1,25-(OH)₂D₃, probably by its action as an oxidant on the trans-plasma-membrane electron-transport sys-

tem, which uses NADH as electron donor [13,14]. Thus cytosolic NADH available in the presence of ascorbate is significantly lower than the levels of NADH observed in 1,25-(OH)₂D₃-induced cells.

Adenylate cyclase of HL-60 cells is inhibited by NADH, an effect that also has been observed in hepatic and adipocyte plasma membranes [29,30]. Therefore the increase in NADH caused by 1,25-(OH)₂D₃ inhibits adenylate cyclase; this inhibition is abolished by the maintenance of lower NADH concentrations by ascorbate, and, as a consequence, more cAMP becomes available for the differentiation process. This mechanism could be responsible for the effect of ascorbate on cAMP levels in hypothalamic cells induced by forskolin or theophylline [47]. However, genomic mechanisms derived from the cAMP increase, such as the increased synthesis of vitamin D receptor reported for dibutyl cAMP in HL-60 cells, should be not discarded [36].

cAMP appears to be an essential factor for the development of the monocytic/macrophagic phenotype of HL-60 cells induced by 1,25-(OH)₂D₃ and the enhancing effect of ascorbate. The differentiation process induced by 1,25-(OH)₂D₃, involving cAMP, may be regulated by cytosolic NADH levels: the decrease in these levels caused by ascorbate and AFR through the plasma-membrane redox system may allow adenylate cyclase to produce more cAMP, which is required for the differentiation process.

This work was supported in part by grants PB95-0560 from the Spanish Dirección General de Enseñanza Superior and FIS no. 96/099. G. L.-LL. is the recipient of a fellowship from Junta de Andalucía. 1α,25-Dihydroxyvitamin D₃ was kindly provided by Solvay-Dupar.

REFERENCES

- Hannah, S. S. and Norman, A. W. (1994) *Nutr. Rev.* **52**, 376–382
- Minghetti, P. P. and Norman, A. W. (1988) *FASEB. J.* **2**, 3043–3053
- Abe, E., Miyaura, C. H., Sakagami, H., Takeda, M., Konno, K., Yamazaki, T., Yoshiki, S. and Suda, T. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4990–4994
- Tanaka, H., Abe, E., Miyaura, C., Shiina, Y. and Suda, T. (1993) *Biochem. Biophys. Res. Commun.* **117**, 86–92
- Kreutz, M. and Andreesen, R. (1990) *Blood* **76**, 2457–2461
- Barton, A. E., Bunce, C. M., Stockley, R. A., Harrison, P. and Brown, G. (1994) *J. Leukocyte Biol.* **56**, 124–132
- Eisman, J. A., Barkla, D. H. and Tutton, P. J. M. (1987) *Cancer Res.* **47**, 21–25
- Saez, S., Falete, N., Guillot, C., Meggouh, F., Lefebvre, M. F. and Crepin, M. (1993) *Breast Cancer Res. Treat.* **27**, 69–81
- Englard, S. and Seifter, S. (1986) *Annu. Rev. Nutr.* **6**, 365–406
- Gershoff, S. N. (1993) *Nutr. Rev.* **51**, 313–326
- Park, C. H., Amare, M., Savin, M. A. and Hoogstraten, B. (1980) *Cancer Res.* **40**, 1062–1065
- Alcaín, F. J., Burón, M. I., Rodríguez-Aguilera, J. C., Villalba, J. M. and Navas, P. (1990) *Cancer Res.* **50**, 5887–5891
- Navas, P., Villalba, J. M. and Córdoba, F. (1994) *Biochim. Biophys. Acta* **1197**, 1–13
- Franceschi, R. T. and Young, J. (1990) *J. Bone Miner. Res.* **5**, 1157–1167
- Quesada, J. M., López-Lluch, G., Burón, M. I., Alcaín, F. J., Borrego, F., Velde, J. P., Blanco, I., Bouillon, R. and Navas, P. (1996) *Calcif. Tissue Int.* **59**, 277–282
- Kobayashi, K., Harada, Y. and Hayashi, K. (1991) *Biochemistry* **30**, 8310–8315
- Crane, F. L., Sun, I. L., Clark, M. G., Grebing, C. and Löw, H. (1985) *Biochim. Biophys. Acta* **811**, 233–264
- Gómez-Díaz, C., Rodríguez-Aguilera, J. C., Barroso, M. P., Villalba, J. M., Navarro, F., Crane, F. L. and Navas, P. (1997) *J. Bioeng. Biomembr.* **29**, 253–259
- Navas, P. and Burón, M. I. (1990) *Oxidoreduction at the Plasma Membrane. Relation to Growth and Transport* (Crane, F. L., Morré, D. J. and Löw, H. E., eds.), vol. 1, pp. 225–236, CRC Press, Boca Raton, FL
- Taetle, R. and Koessler, A. (1980) *Cancer Res.* **40**, 1223–1229
- Chaplinsky, T. J. and Nidel, J. E. (1982) *J. Clin. Invest.* **70**, 953–964
- Bang, B. E., Ericson, C. and Aarbakke, J. (1994) *Pharmacol. Toxicol.* **75**, 108–112
- Yun, K. and Sugihara, H. (1986) *Lab. Invest.* **54**, 336–344
- Yam, L. T., Li, C. Y. and Crosby, W. H. (1972) *Am. J. Clin. Pathol.* **55**, 283–290
- Bradford, M. (1976) *Anal. Biochem.* **72**, 248–252
- Voss, T. and Wallner, E. (1992) *Anal. Biochem.* **207**, 40–43
- Jacobson, E. L. and Jacobson, M. K. (1976) *Arch. Biochem. Biophys.* **175**, 627–634
- Matsumura, H. and Miyachi, S. (1980) *Methods Enzymol.* **69**, 465–470

- 29 Löw, H. and Werner, S. (1976) *FEBS Lett.* **65**, 96–98
- 30 Nair, B. G. and Patel, T. B. (1991) *Life Sci.* **49**, 915–923
- 31 Walters, M. R. (1992) *Endocrine Rev.* **13**, 719–764
- 32 Honda, A., Morita, I., Murota, S.-I. and Mori, Y. (1986) *Biochim. Biophys. Acta* **877**, 423–432
- 33 Bang, B. E., Aarbakee, J. and Sanger, G. (1993) *Scand. J. Clin. Invest.* **53**, 311–315
- 34 Fontana, J. A., Emler, C., Ku, K., McClung, J. K., Butcher, F. R. and Durham, J. P. (1984) *J. Cell. Physiol.* **120**, 49–60
- 35 Bellido, T., Morelli, S., Fernández, L. M. and Boland, R. (1993) *Mol. Cell. Endocrinol.* **90**, 231–238
- 36 Inaba, M., Okuno, S., Koyama, H., Nishizawa, Y. and Morii, H. (1992) *Arch. Biochem. Biophys.* **293**, 181–186
- 37 Inaba, M., Okuno, S., Kogama, H., Ishimura, E., Nishizawa, Y. and Morii, H. (1993) *Nephron* **63**, 152–158
- 38 Savickiene, J., Gineitis, A., Shanbhag, V. P. and Stigbrand, T. (1995) *Anticancer Res.* **15**, 687–692
- 39 Vazquez, G., de Boland, A. R. and Boland, R. L. (1997) *Biochem. Biophys. Res. Commun.* **234**, 125–128
- 40 Muñoz, E., Blázquez, M. V., Ortiz, C., Gómez-Díaz, C. and Navas, P. (1997) *Biochem. J.* **352**, 23–28
- 41 Alcaín, F. J. and Burón, M. I. (1994) *J. Bioenerg. Biomembr.* **26**, 393–398
- 42 Beyer, R. E. (1994) *J. Bioenerg. Biomembr.* **26**, 349–358
- 43 Glascott, Jr., P. A., Tsyganskaya, M., Gilfor, E., Zern, M. A. and Farber, J. L. (1994) *Mol. Pharmacol.* **50**, 994–999
- 44 Villalba, J. M., Navarro, F., Córdoba, F., Serrano, A., Arroyo, A., Crane, F. L. and Navas, P. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 4887–4891
- 45 Burón, M. I., Rodríguez-Aguilera, J. C., Alcaín, F. J. and Navas, P. (1999) *Biochem. Biophys. Res. Commun.* **192**, 439–445
- 46 López-Lluch, G., Burón, M. I., Alcaín, F. J., Rodríguez-Aguilera, J. C. and Navas, P. (1995) *Protoplasma* **184**, 163–167
- 47 Huang, W., Yang, E., Lee, D., Copolov, D. L. and Lim, A. T. (1993) *Endocrinology* **132**, 2271–2273